REMARKS

Support for the amendment to claim 1 can be found, for example, on page 8 of the specification. The amendments to claim 19 are made to clarify the nature of the cells to which the virus is delivered. The amendment to claim 30 simplifies the language of the claim. New dependent claims 33-39 are fully supported by the specification and original claims of the application. Support for new claims 33 - 37 can be found, for example, on page 8 of the specification, and in original claims 3, 5, 6, 9, 11 and 12. New claims 38 and 39 are supported, for example by original claims 19, by pages 10 - 11 of the specification and by the examples presented in the application. No new matter is introduced by these amendments.

Claims 1-28 and 30-32, all of the claims currently pending in the application, remain rejected under 35 U.S.C. § 112, first paragraph, on the basis that the specification does not provide enablement for a vector comprising a ligand other than transferrin non-covalently bound to a virus expressing a gene other than p53, a method for providing said vector to an animal for treating various cancers alone or in combination with radiation or chemotherapy, or a method of providing a vector comprising a ligand bound to a virus expressing the p53 gene to an animal for treating a brain tumor with or without combination

with radiation or chemotherapy. He further asserted that the specification only provides data from animal models and that there is a difference between animal models and human beings. He asserted that the specification fails to provide adequate guidance and evidence for the delivery of the claimed vectors to a human for treating various cancers and that the delivery would result in sufficient expression of the therapeutic product so as to provide a therapeutic effect in the human. Finally, he argued that the specification also fails to provide adequate guidance and evidence for how to deliver the claimed vector comprising any of a variety of different vectors comprising any of a variety of different cell-targeting ligands and therapeutic agents such that the delivery would result in a therapeutic effect in the recipient. The examiner concluded that vector targeting to desired tissues remains unpredictable and inefficient and that it thus would have required undue experimentation to practice over the full scope of the claimed invention. This rejection is traversed.

Although the examiner discussed all of the pending claims together in making this rejection, Applicants believe it will be helpful to address the claims to the composition and the method of making the composition (claims 1-18 and 33-36) and the claims

to the method of treatment (claims 19-28, 30-32, 38 and 39) separately, and the discussion below is so arranged.

The vectors and method of making them

On page 2 of the Office Action, the examiner concedes that the application is enabling for vectors comprising transferrin as the ligand non-covalently and directly bound to an adenovirus comprising a nucleic acid encoding p53. There can be no doubt as to this, as the application provides examples detailing the preparation of such a vector. Similarly, the application describes the preparation of vectors comprising transferrin bound to an adenovirus that comprises a nucleic acid encoding Lac Z. See Examples 2, 7 and 10. In addition, Examples 6 and 8 detail vectors comprising the same ligand and nucleic acid, but comprising a retrovirus or herpes simplex virus, respectively, rather than an adenovirus.

In addition, in a Declaration submitted with the Amendment filed on December 10, 2002, Applicants described the preparation of vectors in which molecules other than transferrin, namely a single chain antibody fragment (TfRscFv) and a protein (epidermal growth factor (EGF)), were used as the ligand.

Applicants thus have provided evidence of making vectors comprising three different types of targeting ligands, two

different genes and three different viruses. The three viruses represent three very different types of viruses, having different sizes and mechanisms of action. These viruses are representative of three broad classes of viruses: adenoviruses represent small, non-enveloped, double-stranded DNA viruses, 70-80 nm in diameter; retroviruses carry RNA, rather than DNA, as their genome along with the mechanism for transcribing DNA from RNA; and Herpes simplex virus is representative of the class of Herpoviridae, which are large, enveloped, DNA viruses with an overall diameter of 150 nm. Thus, these three examples exemplify different size ranges, type of genome and coating (presence or absence of an envelope) and are representative of many other viruses in the same family and/or classification. Indeed, these three viruses are quite representative of the other types of viruses listed in the application and set forth in claim 7 to further exemplify the broad range of the present invention. Adeno-associated viruses are related to adenoviruses. They, too, are small, non-enveloped, DNA viruses. Cytomegalovirus is in the same family as the Herpes simplex virus; it is a large, enveloped, DNA virus. Vaccinia viruses are pox viruses, which, like the Herpes simplex virus, are large, enveloped DNA viruses. Fowlpox virus and canary pox virus also are, as their names indicate, pox viruses. Sindbis virus is a small, RNA virus. Given these illustrations,

it is reasonable and appropriate to conclude that vectors in accordance with claims 1-18 and 33-37 and comprising other viruses can be prepared.

It also is reasonable and appropriate to conclude that vectors comprising other ligands can be constructed. As described on page 8 of the application, useful ligands are proteins, peptides, hormones, antibodies and antibody fragments which specifically target the viral vector to cells which contain receptors for the ligand or which can internalize the ligand by receptor-mediated endocytosis. Selection of a suitable ligand is a matter of routine experimentation based upon the nature and characteristics of the target cells of interest. The scientific literature contains numerous papers which describe receptors over-expressed on various types of cancer cells. Much research has been done to characterize different cancer types in recent years. For example, it is well-accepted that the EGF receptor is over-expressed on breast, ovarian and head and neck cancers, thus making EGF a desirable targeting ligand for the targeted delivery of a virus comprising a therapeutic agent for the treatment of these cancers. Similarly, it is well-known to persons of skill in the art that estrogen receptors are over-expressed on certain types of cancer cells. If one wishes to target such cells, estrogen can be used as the targeting ligand. Alternatively, if

one wishes to target angiogenesis resulting from tumor cell growth, it is known that angiogenesis is associated with the over-expression of the FGF receptor, making FGF a desirable targeting ligand. In addition, receptors for various antibodies are known to be over-expressed on certain types of cancer cells. Antibodies for many of these receptors are commercially available and thus easily obtained.

If one wishes to focus on a cancer less fully characterized in the literature, only routine experimentation is required to determine useful ligands for targeting a viral vector to the particular cancer cell. As noted above, Western blot analyses, which are a standard and routine type of test, can be carried out to determine if receptors to a ligand of interest are over-expressed on a certain type of cell.

Similarly, the application enables the making of the viral-based vectors of the invention. The ligand is bound to the virus through simple admixing in a suitable vehicle, such as sterile water for injection, routine to persons of ordinary skill in the art. The examples of the application well illustrate the preparation of vectors in accordance with the present invention. The examiner has cited no authority as support for a contention that these teachings are insufficient for one of ordinary skill in the art to make other vectors.

Applicants thus respectfully submit that claims 1-18, and new claims 33-37 are enabled by the teachings of the specification.

Methods of Providing therapeutic nucleic acid to cells

Claim 19 is directed to a method of providing a therapeutic nucleic acid to an animal suffering from one of a number of specific types of cancer by administering to the animal a therapeutically effective amount of a vector for delivery of a virus comprising the therapeutic nucleic acid to the cancer cells, wherein a cancer cell-targeting ligand is directly and non-covalently bound directly to the virus and the cancer cells contain a receptor for the ligand. Dependent claims focus on preferred methods of administration, types of viruses, ligands, and therapeutic nucleic acids. In preferred embodiments, the ligand is transferrin and the therapeutic nucleic acid encodes p53. Claims 25, 26, 30-32 and 39 focus on one or both of these preferences. These claims will be discussed first below, followed by a discussion of the broader claims.

Two initial points need to be made, however, which apply to all of the method claims. First, the Applicants wish to make clear that they are not seeking to patent the viral delivery of a therapeutic agent to cancer cells *per se*. Applicants' invention

is a simplified and efficient targeting method for getting a virus to a tumor site, and Applicants' claims are so directed. The art contains many references to the administration of viral vectors containing therapeutic agents; Applicants' invention provides a way to more effectively deliver such vectors to the cells of interest. In the present claims, the cells of interest are certain types cancer cells.

Second, this rejection of the claims is based in part on the examiner's concern, set forth in the paragraph beginning at the top of page 5, that the application fails to provide adequate guidance for how to treat a brain tumor in an animal. Applicants respectfully submit that this concern is irrelevant to the pending claims. All of the method claims are limited to a method of providing a therapeutic agent to an animal suffering from specific cancers, none of which is brain cancer. The specific cancers set forth in claim 19 are head and neck cancer, bladder cancer, breast cancer, thyroid cancer, ovarian cancer, prostate cancer, melanoma or lymphoma.

Turning now to the other specific points raised by the examiner, beginning on page 6 of the outstanding Action, the examiner asserts that claim 19 specifies that the animal to be treated is human and that the application fails to provide adequate guidance for how to provide a therapeutic effect in a

human. He argues that the experimental data of animal models cannot be extrapolated to human studies, and that there is no evidence that delivery of a claimed vector to a human for treating any cancer would provide a therapeutic effect. He also asserted that the specification fails to provide adequate guidance for how to deliver the claimed vector such that it is effective via various administration routes and fails to provide guidance as to which types of tumors contain a transferrin receptor or other claimed ligand receptors. He further asserted that there is insufficient guidance for the correlation between a specific therapeutic nucleic acid and a particular cancer type such that delivery of the nucleic acid can provide a desired therapeutic effect for treatment of that cancer.

As an initial point, Applicants note that claim 19 is not, contrary to the examiner's statement, limited to a method of administration to humans. The claim encompasses the treatment of humans, but the claim does not specify that humans are to be treated.

As noted above, in a preferred embodiment, the therapeutic nucleic acid encodes p53. This embodiment is specifically set forth in claims 25, 30-32 and 39. It is well-established that the administration of a virus carrying a nucleic acid encoding p53 does have a therapeutic effect. See, for example, Shimada,

H., et al., *J. Gastroenterol.* 37 (Supp. 14): 87-91 (2002); Swisher, S.G. et al., *Clin. Canc. Res.*, *9*:93-101 (2003); Swisher, S.G. and J.A. Roth, Curr. Oncol. Rep., 4(4):334-40 (2002; Makower, D., et al., *Clin. Cancer Res.* 9:693-702 (2003); and Pagliaro, L.C., et al., *J. Clin. Oncol.* 21(12):2247-53 (2003). Copies of these papers or abstracts thereof are enclosed. See, also, U.S. Patent 6,410,010, cited by the examiner in this Office Action. A number of viral vectors comprising nucleic acid encoding p53 are in Phase II and Phase III clinical trials for the treatment of cancers. It also is well-established in the art that the p53 gene is non-functional in over 50% of human tumors and is, therefore, a primary target for gene replacement therapy. See, for example, Chada, S., et al., *Curr. Opin. Drug Discov. Devel.* 6(2):169-173 (2003), an abstract of which is enclosed.

Thus, contrary to the examiner's assertion, there is indeed evidence, in the form of papers published in respected scientific journals, that the delivery of a viral vector comprising a therapeutic nucleic acid to a human can provide a therapeutic, anti-cancer effect. Many of these current treatment methods, such as those described in the papers referenced above, have sought to target the viral delivery system through chemical conjugation of a target molecule to the virus, usually through a linker molecule. The present invention represents an advance

over such methods. Not only is the targeting entity not chemically conjugated to the virus, but no linker molecule is involved. Instead, a targeting ligand is directly and non-covalently linked to the virus. The Examples of the application show that this method increases transfection efficiency, which, in turn, will increase the anti-tumor effect of the therapeutic nucleic acid. Again, Applicants wish to point out that their invention is not the administration of a therapeutic nucleic acid-containing viral vector, *per se*; their invention lies in a method for more efficiently targeting such vectors to the cells of interest.

Claims 26, 30-32 and 39 provide that the targeting ligand is transferrin. The examiner asserted that there was insufficient guidance in the application as to which types of tumors over-express transferrin receptor or other ligand receptors. Applicants respectfully submit that they did not need to discuss this, as it is well known in the art that most tumors over-express the transferrin receptor. Transferrin is well-accepted as a tumor targeting ligand. See, for example, Singh, M., *Curr. Pharm. Des.* 5(6):443-451 (June, 1999) ("Transferrin has been used as a ligand for delivering anti-cancer drugs or drug-containing liposomes mostly due to the increased number of transferrin (trf) receptors found on tumor cells as compared to normal cells.");

Thorstensen, K. and I. Romslo, *Scand. J. Clin. Lab. Invest.* 53 (Suppl. 215):113-120 (1993) ("Histochemical analysis of the presence and abundancy of the transferrin receptor will continue to serve as an additional tool in special cases to distinguish between malignant and normal cell growth."); and Yang, D.G., et al., *Anti-cancer Res.*, 21:1777-1788 (2001) ("Tumor cells in a highly proliferative state have a high density of transferrin receptors."), copies of which previously have been provided. One of skill in the art could readily determine if a particular cancer of interest over-expresses the transferrin receptor, such that transferrin would be a suitable choice for the targeting ligand in a viral vector in accordance with this invention. All of the cancer types listed in claim 19 over-express the transferrin receptor.

The examiner expressed concern that gene therapy is unpredictable because of the unpredictability and inefficiency of targeting vectors to the desired tissues or cells. It is exactly that concern which the present invention addresses. Applicants have found a simple and efficient way to target viral vectors containing a therapeutic agent to target cells.

The examiner has argued that experimental data from animal models cannot be extrapolated to human studies. Although the *in vivo* experiments provided in the application were carried out in

mice, in all but Example 7 the mice were ones in which xenografts, i.e., human tumors, had been induced. The use of xenograft-induced mice is, in fact, the standard model in the field of cancer treatment, as evidenced by the website for the National Cancer Institute (NCI), an entire section of which focuses on mouse models. The NCI also has a collaborative program, the NCI Mouse Models of Human Cancers Consortium (MMHCC), and sponsors a variety of other projects to "develop, analyze and apply mouse cancer models." In addition, as has been noted above, the present invention is not the development of a new therapeutic agent *per se* but a method for targeting a therapeutic agent to the specific cells to be treated. The examiner has not provided any evidence that a delivery system which targets human tumor cells present in a mouse model will not also target the same tumor cells present in a human, especially when a receptor for the target ligand is well-known to be over-expressed on those human tumor cells.

Applicants respectfully submit that claims 25, 26, 30-32 and 39 are enabled by their specification. It is known in the art that the p53 gene is nonfunctional in a majority of human tumors, it is known that all of the types of cancer which are the focus of the claims over-express the transferrin receptor, it is known that p53 is non-functional in all of these types of cancers, and

it is known that viral vectors comprising the p53 gene are therapeutically useful, as evidenced by the data being obtained in the various Phase II and Phase III clinical trials currently ongoing. Applicants have presented data which show that viral vectors in which transferrin is directly and non-covalently bound to the virus do efficiently target cancer cells and have efficacy. One of ordinary skill in the art thus could carry out the invention set forth in these claims without engaging in undue experimentation.

Applicant further submit that the remaining method claims, claims 19-24, 27-28 and 38 also are enabled. As described on pages 12-13 above, it would be a matter of routine for one of ordinary skill in the art to determine other useful targeting ligand for a viral vector comprising a therapeutic nucleic acid. Receptors over-expressed on various cancers are known and well-described in the literature; others can be determined through routine experimentation, such as Western Blot tests. Once the ligands are selected and bound to a vector of interest, the vector can be administered such that the ligand targets its receptor on the cancer cells.

In arguing the unpredictability of targeting vectors to target cells, the examiner relied in part upon a 1996 reference by Eck et al. Applicants note that all of the authors' comments

highlighted by the examiner refer to unliganded, non-directed viral vectors. In contrast, the complexes of the present application are tumor-directed, thus overcoming the problem. Applicants have shown through their examples that vectors of their invention do target cancer cells in both *in vitro* tests and in human xenograft mouse models. Applicants also have illustrated intravenous and intratumoral delivery of the vectors, and other delivery methods will be known in the art from current methods used to administer viral vectors carrying therapeutic agents. Applicants thus respectfully submit that the pending claims are enabled.

Claims 1-4, 6, 8-11, 17 and 18 have been rejected under 35 U.S.C. §102(b) as anticipated by Douglas et al., *Intl. J. Oncol.* 11:341-348 (1997). The examiner asserted that the reference teaches the generation of an adenovirus complex through the conjugation of a ligand, folate, to the neutralizing Fab fragment of an anti-knob monoclonal antibody and that the conjugate was complexed with an adenovirus which can be used to deliver a therapeutic gene in cancer gene therapy.

Claim 1, which defines the vector for delivery of a virus to a target cell within a host animal, explicitly sets forth that the vector comprises a cell-targeting ligand and a virus, wherein

the ligand is non-covalently bound directly to the virus. This is in contrast to the delivery system of Douglas et al., who describe a ligand and virus complex in which the ligand is bound to the virus through the neutralizing Fab fragment of a monoclonal antibody. The present application specifically teaches that the direct conjugation of the complexes of the present invention are advantageous in comparison to previously made complexes precisely because they avoid the harsh chemicals and complicated processing steps necessary when the ligand is bound to the virus through a monoclonal antibody, linker, polylysine or other molecule (see, e.g., page 9, lines 6-10, of the specification). The vector of claim 1 and the claims dependent upon it thus represent an advance over prior art vectors, such of that taught by Douglas et al., which require a linker between the virus and the ligand.

The other independent claim of the application rejected as anticipated by this reference, claim 17, directed to a method of making a vector comprising a ligand and a virus for the delivery of a virus to a target cell, also explicitly requires that the ligand be non-covalently directly bound to the virus. This claim thus also is novel over the teachings of Douglas et al.

Claims 1-4, 6, 8-10, 12, 17 and 18 have been rejected under 35 U.S.C. §102(e) as anticipated by U.S. Patent 5,994,109, issued

to Woo et al. (hereinafter referred to as the '109 patent). The examiner asserted that the '109 patent teaches nucleic acid transporter systems for the delivery of nucleic acid to cells, the transporter system comprising a binding complex, which in turn comprises a binding molecule which non-covalently binds to the nucleic acid and covalently links to a surface ligand, nuclear ligand or lysis agent. The binding molecule, such as a cationic peptide, can bind non-covalently to nucleic acid, such as viruses, and also can covalently link to a ligand. The examiner asserted that the cationic peptide can be considered a ligand, which non-covalently can bind to viruses, and it can bind to other ligands for cell-specific delivery of nucleic acid. This rejection is traversed.

As discussed above, both of independent claims 1 and 17 specifically require that the ligand is directly and non-covalently bound to the virus. The vectors of the present invention thus are simple and elegant two part systems. In contrast, the systems disclosed in the '109 patent are all three molecule systems, comprising a ligand, a binding molecule and DNA. More specifically, the '109 patent describes the binding complexes of the invention as comprising a binding molecule which noncovalently links to a nucleic acid of interest to be delivered to a cell and <u>covalently</u> links to a surface ligand, nuclear

ligand and/or a lysis agent (see, for example, the Abstract and the last full paragraph of column 4 in the Summary of the Invention). The '109 patent does not teach or suggest directly and non-covalently binding a ligand to a virus. The patent teaches two uses of a virus. In some examples, an adenovirus is co-transfected with the binding complex. In these instances, the virus does not serve to provide the nucleic acid to be delivered to target cells but as a lysis agent. Alternatively, in the example beginning at line 21 of column 78, the patent teaches a complex comprising DNA, folate and an adenovirus in which the adenovirus is conjugated to a polylysine which, in turn, is conjugated to another polylysine to which folate has been chemically conjugated. The example provides that the adenovirus was attached to the polylysine "under conditions which inactivates the adenovirus binding domain for its receptor." Thus, again, the adenovirus does not serve as the source of the nucleic acid to be delivered to the target cells. The complex made in this example is similar to that described in the example beginning at column 82.

The teachings of the '109 patent thus do not anticipate or render obvious the vector of the present invention nor a method for making it, as the patent does not teach or suggest

compositions in which a virus is directly and non-covalently bound to a cell-targeting ligand.

Claims 1-4, 6-10 and 19-24 remain rejected under 35 U.S.C.§102(e) as anticipated by U.S. Patent 5,962,311, issued to Wickham et al. (hereinafter referred to as the '311 patent). The examiner characterized the patent as teaching a recombinant virus comprising a short-shafted fiber to increase the specificity of binding and a method of targeting the recombinant virus to a cell by contacting the virus vector with a bispecific or multispecific binding agent that selectively binds by non-covalent interaction. This rejection is traversed.

The '311 patent teaches genetically engineering an adenovirus to have a shorter than normal shaft. The shaft is an inherent, normal part of a virus that provides the means by which the adenovirus attaches to its receptor on a cell. As the shaft is a part <u>of</u> the virus, it is not a ligand bound non-covalently <u>to</u> the virus.

The '311 patent requires modifying a virus such that it can target a desired target cell, quite different from the present invention. More specifically, the patent teaches modifying the knob portion of the fiber shaft. The modification results in a reduced level or efficiency of the fiber's binding to its cell surface receptor while binding of a penton base of the virus to

its cell surface receptor is increased. This is said to increase the specificity of the binding of the virus to a given cell. Alternatively, the virus can be targeted to a cell-surface receptor of interest by introducing a non-native amino acid sequence into either the penton base or the fiber knob of the virus. This non-native amino acid sequence is described as comprising a bispecific or multispecific binding agent. This is very different from the present invention.

The patent does not teach or suggest simple direct and non-covalent binding to a virus of a ligand which recognizes a receptor on a target cell such that the virus can be targeted to the target cell, as is required by claim 1 of the present application. Neither, therefore, does the patent teach a method for providing a therapeutic nucleic acid to an animal suffering from certain types of cancers by administering to the animal a virus which comprises the therapeutic nucleic acid and is directly and non-covalently bound to a targeting ligand, wherein the targeting ligand targets a receptor on the cancer cells such that the nucleic acid is targeted to the cancer cells, as required by claim 19 of the present application. The '311 patent thus does not anticipate the claimed invention.

Claims 5, 25, 27 and 28 remain rejected under 35 U.S.C. § 103(a) as obvious over the '311 patent in view of U.S. Patent 6,410,010 issued to Zhang et al. (hereinafter referred to as the '010 patent). The examiner has asserted that although the '311 patent does not teach using a viral vector to encode the p53 tumor suppressor gene, the '010 patent teaches using an adenovirus encoding p53 for gene therapy for cancer cells with aberrant p53 functions and that it would have been obvious to use a vector in accordance with the '311 patent to deliver the p53 gene for gene therapy to cancer cells given the teachings of the '010 patent. This rejection is traversed.

As discussed above, the '311 patent does not teach or suggest directly and non-covalently binding a ligand to a virus. The short-shafted fiber which is the focus of the '311 patent is a modified form of a native component of an adenovirus, not a moiety which is non-covalently bound to a virus. The '010 patent does not compensate for this basic deficiency of the primary reference; it focuses simply on recombinant adenoviruses which carry a nucleic acid encoding p53 under the control of a CMV immediate early promoter. The '010 patent does not teach non-covalently and directly binding a ligand to the virus that will target cells of interest. Nor do the two references, therefore, whether taken independently or together, teach or suggest a

method for providing a therapeutic nucleic acid to an animal suffering from particular types of cancer by administering an effective amount of a vector comprising a virus which comprises the therapeutic nucleic acid and is directly and non-covalently bound to a ligand which targets the cancer cells due to the presence of a receptor for the ligand on the cancer cells. As has been stated above, the present invention is the discovery that by directly and non-covalently binding a ligand which recognizes a receptor on target cells, such as certain types of cancer cells, one can efficiently target a known therapeutic agent, carried in a virus, to the target cells, thereby enabling the therapeutic agent to reach its intended goal. As the cited references do not teach or suggest binding such a ligand to a virus, they do not render the claims of this application obvious.

In view of the foregoing amendments and arguments, Applicants respectfully submit that the claims pending in this application are in condition for allowance.

☒ Customer Number or Bar Code Label **6449**					
Name	Barbara G. Ernst, Reg. No. 30,377				
Signature	Barbara G Ernst			*Date*	Aug. 12, 2003
Address	Rothwell, Figg, Ernst & Manbeck Suite 800, 1425 K Street, N.W.				
City	Washington	*State*	D.C.	*Zip Code*	20005
Country	U.S.A.	*Telephone*	202-783-6040	*Fax*	202-783-6031

1: J Gastroenterol. 2002 Nov;37 Suppl 14:87-91. Related Articles, Links

p53 gene therapy for esophageal cancer.

Shimada H, Matsubara H, Ochiai T.

Department of Academic Surgery, Chiba University Graduate School of Medicine, Chiba 260-8670, Japan.

Despite improvement of surgical treatment and application of multimodality therapies to advanced esophageal cancer, the prognosis is extremely poor for patients with unresectable tumors. Based on the genetic background of esophageal cancer, we have developed various gene therapy strategies against human esophageal cancer. In this article, we review molecular events of esophageal cancer and p53 gene therapy approaches for its treatment. First, we analyzed p53 genetic alterations and angiogenesis in esophageal cancer. Second, we tested a p53 recombinant adenoviral vector (Ad5CMV-p53). Significant growth suppression was observed following infection with Ad5CMV-p53 in human esophageal cancer cell lines. This observation suggests that Ad5CMV-p53 may be a potentially effective therapeutic agent for locally advanced esophageal cancer. Promising avenues for investigation include double gene therapy and adjuvant use of gene therapy with radiation therapy. Third, based on recent reports of clinical trials of p53 gene therapy for lung cancer and head and neck cancer, we developed a clinical protocol for p53 gene therapy for unresectable advanced esophageal cancer. This clinical trial was planned to evaluate vector tolerability and efficacy. Up to December 1, 2001, four patients were enrolled in this phase I/II trial. No serious adverse events related to Ad5CMV-p53 have occurred so far in these patients, and the trial has been safely conducted.

Publication Types:
- Review
- Review, Tutorial

PMID: 12572873 [PubMed - indexed for MEDLINE]

Induction of p53-regulated Genes and Tumor Regression in Lung Cancer Patients after Intratumoral Delivery of Adenoviral p53 (INGN 201) and Radiation Therapy[1]

Stephen G. Swisher,[2] Jack A. Roth, Ritsuko Komaki, Jian Gu, J. Jack Lee, Marshall Hicks, Jae Y. Ro, Waun K. Hong, James A. Merritt, Kamaran Ahrar, N. Edward Atkinson, Arlene M. Correa, Marcelo Dolormente, Linda Dreiling, Adel K. El-Naggar, Frank Fossella, Rhodette Francisco, Bonnie Glisson, Susan Grammer, Roy Herbst, Armando Huaringa, Bonnie Kemp, Fadlo R. Khuri, Jonathan M. Kurie, Zhongxio Liao, Timothy J. McDonnell, Rudolfo Morice, Frank Morello, Reginald Munden, Vassiliki Papadimitrakopoulou, Katherine M. W. Pisters, Joe B. Putnam, Jr., Arcenio J. Sarabia, Thomas Shelton, Craig Stevens, Daniel M. Shin, William R. Smythe, Ara A. Vaporciyan, Garrett L. Walsh, and Min Yin

Section of Thoracic Molecular Oncology, Department of Thoracic and Cardiovascular Surgery[S. G. S., J. A. R., J. G., A. M. C., M. D., R. F., J. B. P., A. J. S., W. R. S., A. A. V., G. L. W., M. Y.], Department of Radiation Therapy [R. K., Z. L., C. S.], Department of Diagnostic Imaging [M. H., K. A., F. M., R. Mu., T. S.], and Department of Pathology [J. Y. R., A. K. E-N., B. K.], and Department of Thoracic/Head and Neck Medical Oncology [W. K. H., F. F., B. G., R. H., F. R. K., J. M. K., R. Mo., V. P., K. M. W. P., D. M. S.], Department of Pulmonary Medicine [A. H.]; Department of Molecular Pathology and Research [T. J. M.], The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030; Introgen Therapeutics, Inc., Houston, Texas 77030 [J. A. M.]; Department of Biomathematics [J. J. L., N. E. A.]; Aventis, Bridgewater, New Jersey [L. D.]; Biotechwrite: Biomedical and Science Communications, Houston, Texas 77079 [S. G.];

ABSTRACT

Purpose: **We designed a prospective single arm Phase II study to evaluate the feasibility and mechanisms of apoptosis induction after *Ad-p53* (*INGN 201*) gene transfer and radiation therapy in patients with non-small cell lung cancer.**

Experimental Design: **Nineteen patients with nonmetastatic non-small cell lung cancer who were not eligible for chemoradiation or surgery were treated as outpatients with radiation therapy to 60 Gy over 6 weeks in conjunction with three intratumoral injections of *Ad-p53* (*INGN 201*) on days 1, 18, and 32.**

Results: **Seventeen of 19 patients completed all planned radiation and *Ad-p53* (*INGN 201*) gene therapy as outpatients. The most common adverse events were grade 1 or 2 fevers (79%) and chills (53%). Three months after completion of therapy, pathologic biopsies of the primary tumor revealed no viable tumor (12 of 19 patients, 63%), viable tumor (3 of 19 patients, 16%), and not assessed (4 of 19 patients, 21%). Computed tomography and bronchoscopic findings at the primary injected tumor revealed complete response (1 of 19 patients, 5%), partial response (11 of 19 patients, 58%), stable disease (3 of 19 patients, 16%), progressive disease (2 of 19 patients, 11%), and not evaluable (2 of 19 patients, 11%). Quantitative reverse transcription-PCR analysis of the four *p53* related genes [*p21* (*CDKN1A*), *FAS*, *BAK*, and *MDM2*] revealed that Bak expression was increased significantly 24 h after *Ad-p53* (*INGN 201*) injection and levels of *CDKN1A* and *MDM2* expression were increased over the course of treatment.**

Conclusions: **Intratumoral injection of *Ad-p53* (*INGN 201*) in combination with radiation therapy is well tolerated and demonstrates evidence of tumor regression at the primary injected tumor. Serial biopsies of the tumor suggest that *BAK* gene expression is most closely related to *Ad-p53* (*INGN 201*) gene transfer.**

INTRODUCTION

Many genes involved in signal transduction, cell cycle control, and apoptosis have been implicated in the etiology of

[1] This work was partially supported by grants from the National Cancer Institute and the National Institutes of Health Grants 01 CA78778-01A1 (to J. A. R.) and SPORE 2P50-CA70970-04); by gifts to the Division of Surgery from Tenneco and Exxon for the Core Laboratory Facility; by the University of Texas M. D. Anderson Cancer Center Support Core Grant CA 16672; by donations from the Charles Rogers Memorial and Gene Therapy Donor funds; by a grant from the Tobacco Settlement Funds as appropriated by the Texas State Legislature (Project 8); the W. M. Keck Foundation; and sponsored research agreement (SR93-004-1) with Introgen Therapeutics, Inc. J. G. is a University of Texas M. D. Anderson Odyssey Program Fellow supported by the Kimberly-Clark Endowment for New and Innovative Research. The University of Texas and J. A. M. are shareholders in Introgen Therapeutics, Inc., the sponsor of this study. J. A. R. is an advisor and paid consultant to Introgen Therapeutics, Inc.

Received 5/17/02; revised 8/13/02; accepted 8/20/02.

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[2] To whom requests for reprints should be addressed, at The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, #445, Houston, TX 77030. Phone: (713) 792-8659; E-mail: sswisher@mdanderson.org.

cancer (1–3) and with elucidation of the mechanisms of action of the products of these genes, many have become potential therapeutic targets (4). The tumor suppressor gene, *p53*, normally responsible for detecting damaged DNA and either directing repair or committing a cell to apoptosis (programmed cell death), is mutated or otherwise altered in >50% of human cancers, including 40–70% of NSCLCs[3]. Altered *p53* has been associated with poor prognosis in patients with many types of cancers (5–8), and several gene replacement-based therapeutic strategies for cancer are in clinical trials (4). However, the precise mechanisms of apoptosis associated with induction of tumor suppressor gene function in human cancers *in situ* have not been previously identified.

Because of the apparent links between *p53* and apoptosis and between apoptosis and radiation, we extended our previous studies of *Ad-p53* gene therapy in NSCLC and initiated a clinical trial of *Ad-p53* (INGN 201) combined with external beam ionizing radiation. We report successful gene transfer, low toxicity, and evidence of tumor regression. In addition, we examined the effects of *Ad-p53* (*INGN 201*) in combination with radiation on cell function by examining expression of several genes known to be regulated by *p53*: *p21* (*CDKN1A*), *FAS*, *BAK*, and *MDM2*. *BAK* expression, alone, was significantly increased 24 h after injection of *Ad-p53* (*INGN 201*) and thus appeared to be the marker most acutely up-regulated by *Ad-p53* (INGN 201), providing the first demonstration of the induction of an apoptotic pathway by tumor suppressor gene expression in actual human cancers.

MATERIALS AND METHODS

Protocol Approval and Data Analysis. The protocol used in this study was approved by the Biosafety and Surveillance Committees of The University of Texas M. D. Anderson Cancer Center, the Recombinant DNA Advisory Committee of the NIH, and the United States Food and Drug Administration. The authors had full access to all of the data in this study and take complete responsibility for the integrity of the data and the accuracy of the data analysis.

Gene Transfer Vector. *Ad-p53* (*INGN 201*) was supplied by Introgen Therapeutics, Inc. (Houston, TX), in frozen aliquots containing 1×10^{12} vp/ml in PBS containing 10% glycerol. Construction and generation of the vector was reported previously (9). Briefly, a replication defective adenovirus serotype 5 was constructed by replacing the viral E1 region with a *p53* expression cassette consisting of a wild-type *p53* gene flanked by the cytomegalovirus promoter and the SV40 polyadenylation signal (9).

Eligibility Criteria and Treatment Protocol. Patients enrolled in the study had histologically proven nonmetastatic NSCLC (stage I–III) with measurable disease. Patients were ineligible for chemoradiation or surgery because of significant comorbidities, age, or obstructed bronchi. Initial treatment with radiotherapy was judged to be the accepted standard of care. The presence of a *p53* mutation in the tumor was not a requirement for study entry.

Study treatment consisted of intratumoral needle injections of *Ad-p53* (*INGN 201*) on days 1, 18, and 32 of treatment in an outpatient setting. Radiation therapy was administered concurrently over 6 weeks, beginning on day 4, to a total of 60 Gy and was directed at the primary tumor and mediastinal lymph nodes if involved. Vector administration was performed by intratumoral injection of the primary tumor either through a flexible bronchoscope or by CT guided percutaneous needle as previously described (10). Tumors $\geq$ 4 cm in the largest diameter were injected with 10 ml divided into three separate sites, whereas tumors with a diameter of <4 cm were injected in a single site with 3 ml. *Ad-p53* doses were escalated initially in cohorts of three for the first 9 patients (3×10^{11}, 1×10^{12}, and 3×10^{12} vp of *Ad-p53*). All subsequent patients received the highest dose (3×10^{12} vp). Core biopsies were obtained from indicator lesions on days 1, 18, 19, and 32 and 3 months after treatment.

Response and Toxicity. The toxic effects of therapy were evaluated according to the National Cancer Institute's Common Toxicity Criteria (11). An independent Data Monitoring Committee whose members were not affiliated with either the University of Texas M. D. Anderson Cancer Center or the sponsor of the trial assessed response to therapy. Assessments were made of overall response (including metastatic sites) and response of the injected tumor (excluding metastatic disease). The Data Monitoring Committee used standard criteria with CT and bronchoscopic findings and not pathologic biopsies (12). Response of the primary injected tumor focused only on the primary tumor, excluding progression at metastatic sites.

Survival duration was measured from beginning of therapy to date of last follow-up or death. Time to progression for metastatic (pleural effusions, pulmonary nodules, systemic metastases) and locoregional disease (primary tumor and mediastinal or hilar lymph nodes) was defined as the time from beginning of therapy to documented progression. Patients who did not demonstrate progression were censored at the time of last follow-up.

Radiation Therapy. External radiation therapy was given by linear accelerator 18 or 6 Mv with a total dose of 60 Gy calculated at the isocenter in 30 fractions over 6 weeks without inhomogeneity correction. The margins ranged from 2 to 2.5 cm around the gross target volume.

Real-Time PCR and Reverse Transcription-PCR. Probes and primers used in this study were designed using the Primer Express software (version 1.0; Perkin-Elmer). Sequences are available upon request. To avoid amplification of contaminating residual genomic DNA, probe and primer sets for each gene were designed around the junction region of two exons so that they are mRNA-specific.

To determine the copy number of *Ad-p53* virus in each cell, viral DNA extracted from *Ad-p53* was used as an absolute standard, and the β-actin gene was used as a reference gene to count cell numbers. Briefly, calculation of *p53* virus copy number was accomplished by plotting a β-actin standard curve, using human genomic DNA (from Perkin-Elmer) as a standard (1 ng of DNA equals ~303 genome equivalents) and comparing the results of the clinical samples with the standard using

[3] The abbreviations used are: NSCLC, non-small cell lung cancer; vp, viral particles; CT, computed tomography; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; *wt-p53*, wild-type *p53*.

β-actin probes. This resulted in the number of genomes (cells) in each sample. The number of *p53* virus copies in each sample was determined by comparing with a separate *p53*-virus standard curve (plotted using *p53*-viral DNA standard.) This value was corrected for the presence of inflammatory cells.

For quantitative real-time reverse transcription-PCR, human total RNA was used as a relative standard and human *GAPDH* gene served as an internal control for relative mRNA amount. Real-time PCR was performed in the ABI Prism 7700 Sequence Detection System according to the manufacturer's protocol.

Statistical Considerations. The purpose of this nonrandomized Phase I/II study was to evaluate the efficacy of *Ad-p53* (*INGN 201*) gene therapy as an adjunct to radiation therapy in the treatment of patients with NSCLC. The primary end point for evaluation was the local control of tumor at 3 months. The study was designed to test the null hypothesis that the 3-month local control rate is 20% *versus* the alternate hypothesis that the rate is 40% using a one-sided exact binomial test with an α level of 5%. The sample size of 49 patients provided a power of 83%. An interim analysis after 15 patients was included in the design.

Data from 17 of 19 patients enrolled in this study were analyzed. For each patient, the gene expression for *p53* and *GAPDH* were measured in duplicate at four distinct time points: time 0 = baseline, before any therapy; time 1 = on day 18 after the first *Ad-p53* (*INGN 201*) injection and after 2 weeks of radiation therapy but before the second *Ad-p53* (*INGN 201*) injection; time 2 = on day 19, 24 h after the second *Ad-p53* (*INGN 201*) injection and after 2 weeks of radiation therapy; and time 3 = at day 32, before the third *Ad-p53* (*INGN 201*) injection and after 4 weeks of radiation therapy. Ratio of the marker gene and *GAPDH* was calculated to estimate the amount of target gene expression. The coefficient of variation (defined as SD divided by the mean) was computed to estimate the precision of the duplicate experiment. Modulation of gene expression over time was assessed by comparing the ratios of the target gene between two time points. Exact binomial test was applied to test the null hypothesis of no modulation by assuming that the probability of up-regulation (or down-regulation) equals to 0.5. Two-sided *P*s were calculated. Duplicate experiments were done when adequate tissue samples were available (patients 16, 15, 13, 12, and 11 at time 0, 1, 2, and 3, respectively). The coefficient of variation ranged from 0 to 1.21 with a median of 0.2. There were 78 and 97% of the samples with coefficient of variation < 0.5 and 0.8, respectively, indicating good reproducibility between the duplicate experiments. The precision was similar among all four time points.

RESULTS

Patient and Tumor Characteristics. Nineteen patients (8 female, 11 male; median age, 74, age range, 53–91) with nonmetastatic NSCLC who were not eligible for chemoradiation or surgery were enrolled in this Phase II study (Table 1) between April 30, 1998, and May 4, 2000. The date of last follow-up was April 1, 2002, with a median follow-up of 36 months. All patients had histologically determined viable NSCLC on pretreatment tumor biopsies. Nine patients had locoregionally advanced NSCLC (5 stage IIIA and 4 stage IIIB) and were ineligible for chemoradiation because of poor performance status, age, comorbidities, or obstructed bronchi. Ten patients with stage I and II NSCLC (2 stage IA, 5 stage IB, and 3 stage IIB) were ineligible for surgery because of poor pulmonary function tests or significant comorbidities.

Patients were treated with radiation therapy to 60 Gy over 6 weeks, in conjunction with three intratumoral injections of *INGN 201* (on days 1, 18, and 32) via CT guidance (15 patients) or bronchoscopy (4 patients) administered into the primary tumor (for dose assignment see Table 1). Seventeen of 19 patients received all planned treatment, whereas 2 patients did not complete therapy because of tumor progression (patient no. 18) or early death (patient no. 6). Two additional patients did not receive tumor biopsies 3 months after completion of therapy because of tumor progression (patient no. 10) or weakness (patient no. 19). The presence of a *p53* mutation in the primary tumor was not required for entry into the study because previous studies in animal models, as well as in clinical trials, have shown no clear relationship between *p53* mutational status and response to INGN 201 treatment (10, 13–15).

Seventeen of 19 patients completed the radiation therapy according to the protocol with a total tumor dose of 60 Gy in 30 fractions (2 Gy/day) by high energy ($\geq$6 Mv) accelerated photons. The duration of the radiation therapy ranged from 39 to 50 days with the median duration as 44 days. There were no major protocol violations such as prolonged interruption of radiation therapy or administration of nonstudy anticancer therapy among the 17 patients.

Antitumoral Efficacy. Three months after completion of radiation therapy and *Ad-p53* (*INGN 201*) therapy, antitumoral efficacy was determined with CT scan evaluation (16 of 19 patients) and pathologic examination of biopsies (15 of 19 patients). Pathologic examination of biopsies 3 months after completion of therapy revealed no viable tumor in 12 patients (12 of 19, 63%) and viable tumor in 3 of 19 patients (16%). Tumors of 4 patients (4 of 19, 21%) were not biopsied because of tumor progression (patients nos. 10 and 18), early death (patient no. 6), or weakness (patient no. 19). The study was closed to additional accrual after the planned interim analysis after 19 patients.

Assessment of the primary injected tumor 3 months after completion of therapy (Table 1, Fig. 1, *A* and *B*) was performed by an external review board with CT and bronchoscopic findings and demonstrated: a CR in 1 patient (1 of 19, 5%); PR in 11 patients (11 of 19, 58%); stable disease in 3 patients (3 of 19, 16%); and PD in 2 patients (2 of 19, 11%). Two patients (2 of 19, 11%) were nonevaluable because of tumor progression (patient no. 18) or early death on treatment day 69 (patient no. 6).

Overall tumor response (including metastatic disease apparent at the time of posttreatment evaluation) was determined by an external review board, based on CT, bronchoscopic, and clinical findings. CR was seen in 1 patient (1 of 19, 11%), PR in 5 patients (5 of 19, 26%), SD in 1 patient (1 of 19, 5%), and PD in 11 patients (11 of 19, 58%). Six patients progressed locoregionally (4 primary injected tumor alone, 1 primary injected tumor, and mediastinal lymph nodes [lymph nodes irradiated but not injected with *Ad-p53* (*INGN 201*)], 1 mediastinal lymph node alone [not irradiated or injected with *Ad-p53* (*INGN 201*)]. With an intention to treat analysis and median follow-up

Table 1 Characteristics of NSCLC patients and tumors after treatment with intratumoral injection of Ad-p53 and radiation therapy

Patient no.	Ad-p53 dose (viral particles)	Age (yr)	Gender	Histology[a]	Stage	Injection site[b]	Baseline measure of primary tumor (cm^2)	Injected site response[c]	3-month biopsy[d]	Locoreg recur[e]	Distant recur[e]	Overall response[f]
1	3×10^{11}	74	Male	Squam	IIIA	RUL	4 × 4	PR	Pos	No	Yes	PD
2	3×10^{11}	78	Female	NSCLC	IIB	LUL bronchus	4 × 4	PR	Neg	No	No	PR
3	3×10^{11}	69	Male	Squam	IIB	RUL bronchus	3 × 5	CR	Neg	No	No	CR
4	1×10^{12}	73	Female	Adeno	IIIA	RLL	6 × 4	SD	Neg	Yes	Yes	PD
5	1×10^{12}	74	Male	Adeno	IIIB	LUL	8 × 5	PR	Neg	No	Yes	PD
6	1×10^{12}	72	Male	Squam	IIIB	LLL	9 × 7	NE[g]	NE[g]	No	No	PD
7	3×10^{12}	74	Female	Adeno	IB	RUL	4 × 4	PR	Neg	Yes[h]	Yes	PR
8	3×10^{12}	81	Female	Adeno	IIB	RUL	4 × 3	PR	Pos	Yes	No	PR
9	3×10^{12}	68	Male	Adeno	IB	RUL	5 × 5	SD	Pos	No	No	SD
10	3×10^{12}	53	Male	Squam	IIIA	R mainstem bronchus	5 × 7	NE[g]	NE[g]	No	Yes	PD
11	3×10^{12}	73	Male	Adeno	IIIA	LUL	5 × 5	PR	Neg	No	No	PR
12	3×10^{12}	79	Female	Squam	IA	RUL	3 × 2	PR	Neg	No	No	PR
13	3×10^{12}	91	Male	Adeno	IIIB	RUL	6 × 7	PR	Neg	No	Yes	PD
14	3×10^{12}	85	Female	Squam	IB	LLL	2 × 3	SD	Neg	No	Yes	SD
15	3×10^{12}	81	Female	Adeno	IB	RUL	3 × 2	PD[i]	Neg	No	No	PD[i]
16	3×10^{12}	67	Male	Squam	IB	RUL	8 × 5	PR	Neg	Yes[h]	Yes	PD
17	3×10^{12}	58	Female	Squam	IIIB	RUL bronchus	3 × 6	PR	Neg	Yes	Yes	PD
18[j]	3×10^{12}	62	Male	Squam	IIIB	LUL	9 × 9	PD	NE[g]	Yes	Yes	PD
19	3×10^{12}	84	Male	NSCLC	IA	RLL	2 × 3	PR	NE[g]	No	Yes	PD

[a] All tumors histologically confirmed, pretreatment, to be viable NSCLC: squam, squamous cell carcinoma; adeno, adenocarcinoma.

[b] Location of primary tumor injected with Ad-p53 (INGN 201). Tumors located in bronchus injected by bronchoscopy others by CT guidance: RUL, right upper lobe; RLL, right lower lobe; LUL, left upper lobe; LLL, left lower lobe.

[c] Response of injected primary tumor determined 3 months after completion of therapy as determined by external review board. CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; NE, not evaluable.

[d] Pathologic biopsy of tumor obtained 3 months after completion of therapy by CT scan, core needle, or bronchoscopic biopsy.

[e] Locoregional and metastatic recurrence determined by serial bronchoscopy or CT scans. Locoregional recurrence defined as progression in primary tumor or locoregional lymph nodes (mediastinal or bronchial). Metastatic recurrence defined as progression in distant sites, including other organs, pulmonary nodules, and pleural effusions.

[f] Overall response determined 3 months after completion of therapy by external review board. Clinical progression of disease (patient no. 6).

[g] NE, not evaluable because patient died before biopsy (patients nos. 6, 10) or refused biopsy because of disease progression (patients nos. 18, 19).

[h] Mediastinal lymph nodes that were not injected with INGN 201 or in radiation portals increased in size (patient no. 16), whereas the primary tumor that was irradiated and injected with INGN 201 decreased in size. Patient no. 7 developed progression in primary tumor and mediastinal lymph nodes that were both in radiation portals.

[i] PD because CT scan showed increase in size at 3 months but probable radiation change because no tumor progression over subsequent 18 months of CT follow-up and negative pathologic biopsy at 3 months.

[j] Patient no. 18 did not complete INGN 201 and radiation therapy treatment because of clinical progression.

of 37 months, overall survival analysis by Kaplan Meier is 47% at 1 year and 26% at 3 years (Fig. 2*A*). Five patients are currently alive without evidence of disease 34–48 months after the start of treatment. Eleven patients have developed clinically diagnosed distant metastases (5 pleural effusions, 2 pulmonary nodules, 1 bone, 1 brain, 1 brain, and s.c. nodules and 1 adrenal). Median time to progression has not been reached (Fig. 2*B*) for locoregional disease and is 9.2 months for metastatic disease (Fig. 2*C*).

Adverse Events. All patients received radiation therapy and *INGN 201* gene therapy as outpatients. The most common adverse events associated with *Ad-p53* (*INGN 201*) vector administration and radiation were grade 1 or 2 fevers (79%), pain (68%), chills (53%), and pneumothoraces (37%; Table 2). All pneumothoraces were managed on an outpatient basis, with observation or percutaneous catheters. Radiation therapy was associated with primarily grade 1 or 2 esophagitis (47%), anorexia (21%), or weakness (58%). Grade 3 (severe) or grade 4 (life threatening) toxicity was noted in 6 of 19 (33%) patients and consisted of atrial arrhythmias, anemia, weakness, anorexia, pain, nausea, dyspnea, confusion, hypotension, and hallucination. The combination of *Ad-p53* (*INGN 201*) and radiation therapy did not appear to increase toxicity over what was expected with radiation therapy alone based on previous experience (16, 17). No patients discontinued radiation therapy or *Ad-p53* (*INGN 201*) because of treatment-related adverse events. In addition, no treatment related deaths were observed.

Assessment of Gene Transfer by Ad-p53 (INGN 201). The presence of *Ad-p53* vector-specific DNA and mRNA was assessed (Table 3) by quantitative real-time PCR in preinjection (day 18 after entry into the protocol) and 24 h after injection (day 19) biopsy specimens. The β-actin genome number is

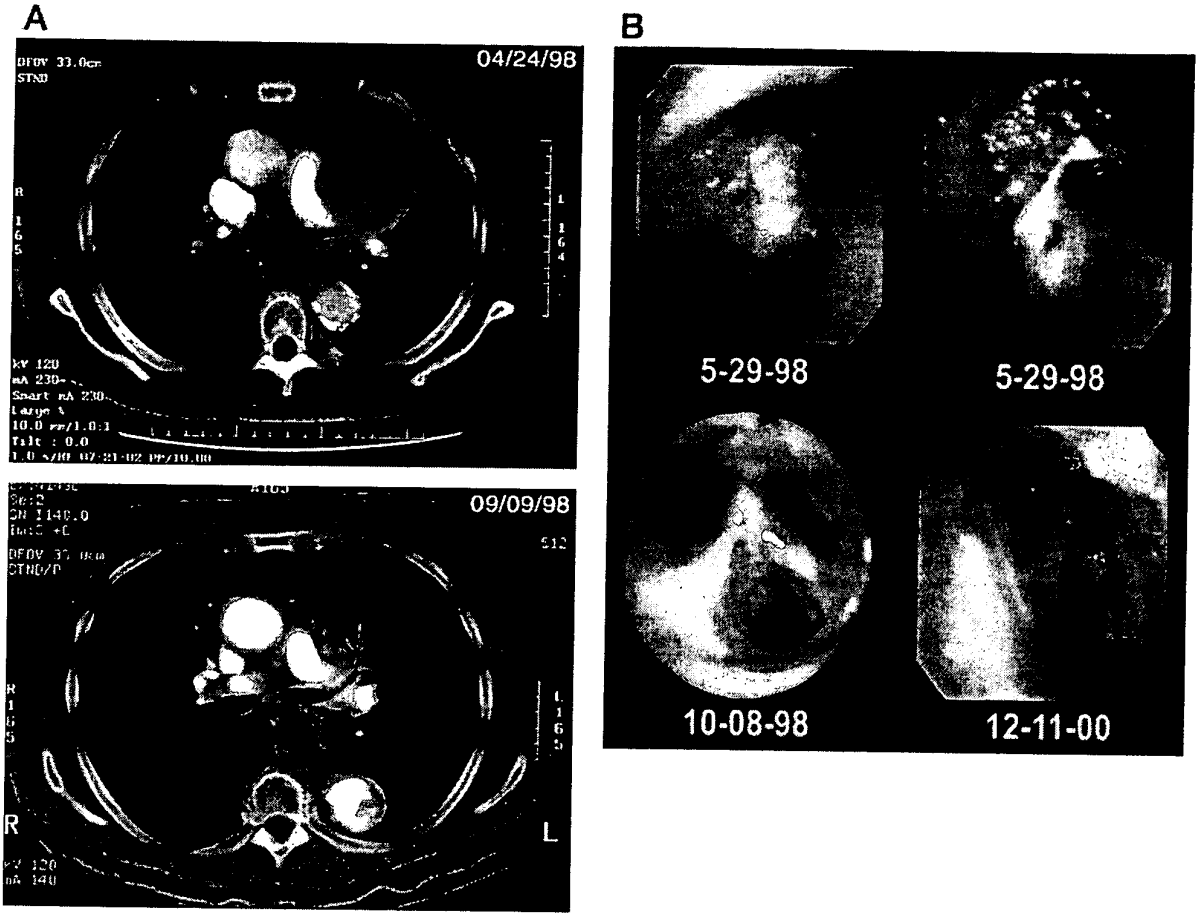

Fig. 1 *A*, patient no. 2: left upper lobe tumor unable to undergo surgery because of poor pulmonary function and cardiac disease. Patient received three injections of *Ad-p53* (3×10^{11} vp) via bronchoscope in combination with radiation therapy (60 Gy; *A*). Pathologic biopsy negative for viable tumor 3 months after completion of therapy (*B*). *B*, patient no. 3: right upper lobe tumor unable to be treated with surgery because of poor pulmonary function and ineligible for chemotherapy because of cardiac disease and obstructed bronchus (5/29/98). Patient was treated with three injections of *Ad-p53* (3×10^{11} vp) and radiation therapy (60 Gy) by bronchoscopy (5/29/98) with a CR 3 months after completion of therapy (10/8/98) and no pathologic evidence of tumor 29 months after therapy (12/11/00).

given in the table in parentheses as a positive control to show the presence of DNA in the sample. All values were corrected for the percentage of nontumor cells present in the biopsy.

Ad-p53 vector-specific DNA was detected in biopsies from 9 of 12 patients with paired biopsies (day 18 and day 19). No *Ad-p53* vector-specific DNA was detected in pretreatment biopsy specimens before the first *Ad-p53* injection (data not shown). The ratio of copies of *Ad-p53* vector DNA to copies of β-actin DNA was 0.15 or higher in 8 of 9 patients (range, 0.05–3.85) with 4 patients having a ratio >0.5. For 11 patients with adequate samples for both vector DNA and mRNA analysis, 8 showed a postinjection increase in mRNA expression associated with detectable vector DNA. Postinjection increases in *p53* mRNA were detected in 11 of 12 paired biopsies obtained 24 h after *Ad-p53* (*INGN 201*) injection, with 10 of 11 increasing 3-fold or greater ($P < 0.005$). Comparison of *Ad-p53* mRNA levels in days 18, 19, and 32 biopsy specimens to pretreatment biopsies also showed a highly statistically significant increase (data not shown; $P < 0.005$). Preinjection biopsies that were negative for *p53* protein expression by immunohistochemistry were stained for *p53* protein expression after *Ad-p53* (*INGN 201*) injection. Staining results confirmed that the *p53* protein was expressed in the posttreatment samples in the nuclei of cancer cells (data not shown).

Effect of *Ad-p53* (*INGN 201*) Gene Transfer on mRNA Expression of *p53*-regulated Genes. Previous *in vitro* experiments in human NSCLC cell lines identified four genes [*p21* (*CDKN1A*), *MDM2*, *FAS*, and *BAK*] that showed the greatest increase in mRNA expression after induction of *p53* overexpression with *Ad-p53* (data not shown). Therefore, in the current study, changes in mRNA levels for these four markers were determined at various time points before and during treatment using reverse transcriptase real-time PCR (Table 4). The study was controlled by obtaining a pretreatment biopsy under the

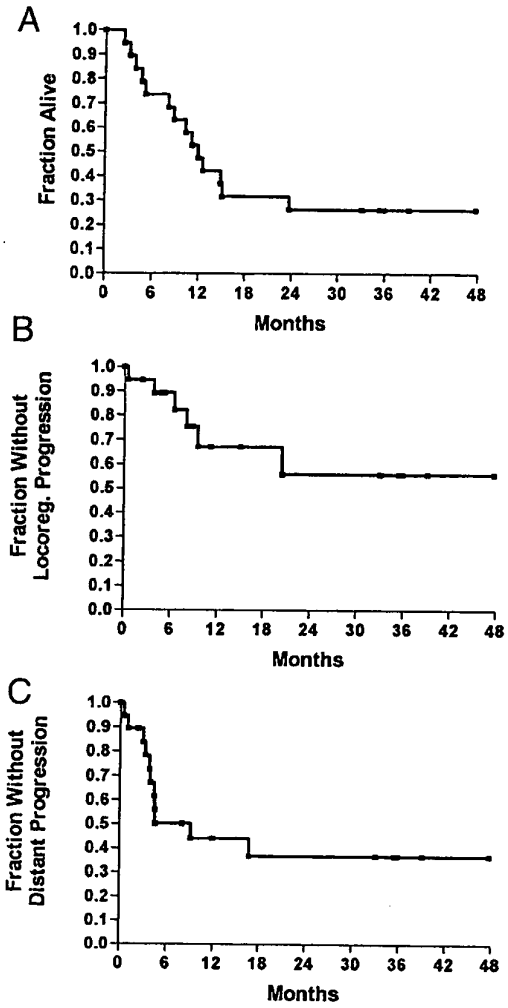

Fig. 2 *A*, overall survival of all patients (n = 19) entered on trial with *Ad-p53* (*INGN 201*) and radiation therapy. *B*, locoregional time to progression of all patients (n = 19) entered on trial with Ad-p53 (*INGN 201*) and radiation therapy. *C*, metastatic time to progression of all patients (n = 19) entered on trial with Ad-p53 (*INGN 201*) and radiation therapy.

same conditions as the posttreatment biopsy. The inclusion of a time point during the radiation treatment allowed for a biopsy to be performed immediately before and 24 h after *Ad-p53* injection, thus allowing determination of the effects of the *Ad-p53* on mRNA expression during treatment. An increase in mRNA expression was defined as a ratio >2 compared with GAPDH; a decrease was defined as a ratio <0.5; any value between 0.5 and 2 was considered no change. The exact binomial two-sided test was used to test the null hypothesis of no modulation between time points. The time intervals in Table 4 refer to: (*a*) change between day 18 (immediately before injection) and day 19 [24 h after *Ad-p53* (*INGN 201*) injection]; and (*b*) change between day 0 (before initiation of all treatment) and days 18, 19, and 32 (days after initiation of treatment).

For *p21* (*CDKN1A*) mRNA, increases of statistical significance were noted 24 h after *Ad-P53* injection (time interval 1, borderline, P = 0.07) and during treatment, as compared with the pretreatment biopsy (time interval 2, P = 0.02). In the case of *MDM2* mRNA, increases of statistical significance were noted during treatment compared with the pretreatment biopsy (time interval 2, P = 0.04). Levels of *FAS* mRNA did not show statistically significant changes during treatment. BAK mRNA expression increased significantly 24 h after injection of *Ad-p53*(INGN 201; time interval 1, P = 0.04) and thus appeared to be the marker most acutely up-regulated by *Ad-p53* (*INGN 201*) injection rather than *Ad-p53* (*INGN 201*) and radiation therapy (time interval 2, P = 0.80). There were too few specimens to find a statistical correlation with gene up-regulation and clinical outcome.

DISCUSSION

Conventional radiation and sequential chemoradiation strategies provide poor locoregional control of NSCLC, with only 15–20% local control at 1–2 years (16, 17). In an effort to determine whether *Ad-p53* (*INGN 201*) could enhance locoregional control, this trial was designed to evaluate not only radiographic responses but also pathologic biopsies 3 months after completion of therapy. A previous study by Le Chevalier *et al.* (16) resulted in a negative pathologic biopsy rate 3 months after completion of radiation or sequential chemoradiation of only 17–20%. Although our study cannot be compared directly with this study, the high number of pathologic negative biopsies (63%, intention to treat) and the large number of radiological responses at the primary tumor site [60% major response (PR or CR), intention to treat] in a patient population that was unable to tolerate chemoradiation or surgery is encouraging. Because survival in locoregionally advanced NSCLC is also dependent on control of metastatic disease, Phase III randomized studies will be necessary to determine whether the potential improvement in locoregional control achieved by *Ad-p53* (*INGN 201*) and radiation therapy can translate into improved overall survival. Our study did, in fact, demonstrate a high metastatic failure rate (Fig. 2*C*), which may have been expected because chemotherapy could not be administered to these high-risk patients. In future studies, we plan to address metastatic relapse by adding chemotherapy to the combination of *Ad-p53* (*INGN 201*) and radiation therapy.

It is encouraging that strategies designed to improve locoregional control in locoregionally advanced NSCLC such as concurrent chemoradiation or fractionated radiation therapy have led to improved survival (17–19). The Japanese Clinical Oncology Group and the Radiation Therapy Oncology Group recently reported improved survival in locoregionally advanced NSCLC when concurrent chemoradiation rather than sequential chemoradiation was used, presumably because of the radiation sensitizing effect of concurrent chemotherapy (19–21), although toxicity appeared increased with concurrent chemotherapy. Additionally, subset analysis of these studies have demonstrated that concurrent chemoradiation might not be as effective in elderly or poor performance status patients, in part, because of increased toxicity (22). These observations suggest a therapeutic window for *Ad-p53* (*INGN 201*) and radiation therapy

Table 2 Highest grade adverse event (AE) observed in 19 patients during *Ad-p53* (INGN 201) and radiation therapy

Adverse events[a]	Grade 1[b,c]	Grade 2[b,c]	Grade 3[b,c]	Grade 4[b,c]	Total patients with AE[d]
Fever	2 (11)	13 (68)	0	0	15 (79)
Pain	7 (37)	5 (26)	1 (5)	0	13 (68)
Weakness	4 (21)	4 (21)	3 (16)	0	11 (58)
Nausea	9 (47)	0	1 (5)	0	10 (53)
Chills	7 (37)	3 (16)	0	0	10 (53)
Esophagitis	6 (32)	3 (16)	0	0	9 (47)
Dyspnea	6 (32)	1 (5)	1 (5)	0	8 (42)
Vomiting	5 (26)	3 (19)	0	0	8 (42)
Pneumothorax	4 (21)	3 (16)	0	0	7 (37)
Arrhythmia	5 (26)	0	0	2 (11)	7 (37)
Hemoptysis	4 (21)	2 (11)	0	0	6 (32)
Anorexia	3 (16)	0	1 (5)	0	4 (21)
Hypotension	0	1 (5)	1 (5)	0	2 (11)
Anemia	0	0	2 (11)	0	2 (11)
Confusion	0	0	1 (5)	0	1 (5)
Hallucination	0	0	1 (5)	0	1 (5)

[a] Adverse events listed as descriptive or verbatim term from medical records, not otherwise coded.
[b] Toxicity defined by National Cancer Institute common toxicity criteria (Grade 1–4); percentage of patients with this level of toxicity in parentheses.
[c] Highest grade toxicity associated with *Ad-p53* (INGN 201) and radiation therapy treatment; percentage of patients with this level of toxicity in parentheses.
[d] Total number of patients treated with *Ad-p53* (INGN 201) and radiation therapy with AE.

Table 3 Detection of *Ad-P53* vector-specific DNA and mRNA in biopsy specimens by real-time PCR

	Ad-p53 DNA PCR *Ad-p53* copy number: β-actin genome[a] (copies of β-actin genome)		*Ad-p53* mRNA reverse transcriptase PCR[a] *p53* mRNA: GAPDH mRNA	
Patient no.	Preinjection[b]	Postinjection[c]	Preinjection	Postinjection
1	neg[d] (68)	neg (112)	218	5308
2	neg (149)	3.85 (92)	<1	23280
3	neg (71)	3.06 (60)	25	81
4	na[e]	na	na	na
5	neg (94)	neg (267)	176	766
6	neg (334)	0.05 (458)	35	170502
7	neg (17)	0.71 (73)	167	11365
8	neg (347)	0.39 (220)	20	33
9	neg (1221)	neg (1666)	<1	2214
10	neg (285)	0.19 (611)	<1	94
11	neg (200)	0.24 (219)	18	220
12	na	na	na	na
13	neg (194)[f]	2.76 (248)	7	701
14	0.16 (408)	neg (421)	na	na
15	na	na	na	na
16	na	na	113	78
17	na	na	na	na
18	na	na	na	na
19	na	na	na	na

[a] Values represent the mean of duplicate samples.
[b] Day 18.
[c] Day 19.
[d] neg, not detectable in biopsy; sensitivity of the quantitative DNA PCR is one copy as determined by standard curves; for the reverse transcription-PCR, the sensitivity is between 10 and 100 copies by quantitation in run-off transcription assays (data not shown).
[e] na, biopsy not available.
[f] Pretreatment biopsy was obtained before day 1 injection.

because this novel strategy demonstrated no dose-limiting toxicity with concurrent use and was limited only by manufacturing considerations. Although these patients were often ineligible for chemoradiation because of age or significant comorbidities, only 34% of the patients suffered a grade 3 or higher adverse event, and all patients were treated as outpatients. In the future, *Ad-p53* (*INGN 201*) may, in combination with chemoradiation, provide enhanced survival by increasing locoregional control without increasing toxicity.

Another major goal of this study was to determine the molecular mechanism by which *Ad-p53* (*INGN 201*) and radiation therapy-induced cell killing. We therefore performed mul-

Table 4 Changes in mRNA levels of marker genes at various time points before and during treatment

mRNA	Time interval[a]	Patients with increased[b] mRNA levels	Patients with decreased[c] mRNA levels	*P* (two-sided)
P21 (CDKN1A)	1	7	1	0.07
	2	11	2	0.02
MDM2	1	4	6	0.75
	2	8	1	0.04[d]
FAS	1	5	6	~1.00
	2	5	3	0.73
BAK	1	8	1	0.04[d]
	2	9	7	0.80

[a] Time interval 1, interval between day 18 [immediately before injection of *Ad-P53* (INGN 201)] and day 19 [24 h after *Ad-p53* (INGN 201) injection]; time interval 2 = change between day 0 (before initiation of any treatment) and days 18, 19, and 32 (days after initiation of treatment).
[b] Two-fold or greater increase over pretreatment value.
[c] Fifty percent or greater decrease from pretreatment value; patient samples not classified as either an increase or decrease are considered unchanged or not available for analysis from a total of 16 possible specimens.
[d] Statistically significant, $P < 0.05$.

tiple biopsies throughout the study to evaluate the time course of the induction of several apoptosis-related genes and their relationship to *p53* gene transfer and radiation therapy. We demonstrated for the first time in human cancers *in situ*, the induction of expression of several genes closely linked to *p53*, including *MDM2*, *p21* (*CDKN1A*), and *BAK*. Our study showed that, although *p21* (*CDKN1A*) and *MDM2* appeared to be modestly up-regulated in tumors injected with *Ad-p53* (*INGN 201*), it was the proapoptotic gene *BAK* that showed significant up-regulation within 24 h of *Ad-p53* (*INGN 201*) injection. Because of the study design, it remains possible that radiation alone had an effect on the marker genes independent of *Ad-p53* (*INGN 201*) administration. A study by Bishay *et al.* (23), however, showed that *BAK* mRNA remains at a constant level in cells with endogenous *wt-p53* after radiation. Thus, our observation of an increase in *BAK* links this specifically to the forced overexpression of *p53* by *Ad-p53* (INGN 201) gene transfer. Previously, Pearson *et al.* (24) showed up-regulation of *BAK* protein expression in lung cancer cell lines in response to forced overexpression of p53. Bishay *et al.* (23) also showed increased expression of *p21* (*CDKN1A*) in B lymphoblastoid cells, suggesting that radiation in the presence of *wt-p53* can induce *p21* (*CDKN1A*) expression. However, in our study, this cannot be specifically attributed to forced overexpression of *p53* because radiation can achieve this with low levels of *wt-p53*. In lung tumors with nonfunctional *wt-p53*, which likely includes most of tumors in our study, the restoration of *wt-p53* function probably contributed to the increases in *p21* (*CDKN1A*) expression.

The role of these genes in mediating tumor regression and apoptosis in patients will require further study. However, previous studies have shown conclusively that forced overexpression of *p53* by both retroviral and adenoviral vectors is associated with marked increases in the apoptotic fraction of NSCLC cells in biopsies taken 72 h after injection of *Ad-p53* as shown by terminal deoxynucleotide transferase-mediated biotin UTP nick-end labeling staining (10, 13, 25). The up-regulation of the proapoptotic gene *BAK* may, in part, be responsible for this effect, although this study cannot determine whether *Ad-p53* was responsible because radiation was administered simultaneously. Although many genes may be under the regulatory control of *p53*, we initially screened several NSCLC cell lines for those apoptosis-associated genes most strongly up-regulated by forced *p53* overexpression. Thus, although our study does not include all known *p53* regulated genes, it provides important confirmation of the cell culture findings and a methodology for future studies.

Future clinical trials will explore the combination of *Ad-p53* (*INGN 201*) gene transfer and chemoradiation to address both metastatic and locoregional disease. The antitumoral potential of these strategies is supported by preclinical data that suggests the combination of all three treatments (*Ad-p53*, chemotherapy, and radiation therapy) is synergistic and may lead to enhanced antitumoral activity without increased toxicity.[4] This study has been an important foundation for future studies because it provides a molecular mechanism for the enhanced antitumoral activity with sequential tumor biopsies and quantitative analysis of *p53*-regulated genes. It confirms that after *Ad-p53* (*INGN 201*) gene transfer and radiation therapy, *wt-p53* gene expression increases dramatically with subsequent induction of *BAK*, *MDM2*, and *p21* (*CDKN1A*). In the future, these molecular markers may help clinicians to identify those patients most likely to respond to *Ad-p53* (*INGN 201*) gene transfer strategies.

ACKNOWLEDGMENTS

We thank Dr. Wafik El-Deiry at the Howard Hughes Medical Institute, University of Pennsylvania for his helpful comments on the manuscript.

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p53 Gene therapy for lung cancer.

Swisher SG, Roth JA.

Department of Thoracic and Cardiovascular Surgery, The University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Boulevard, Box 445, Houston 77030, USA. sswisher@mdanderson.org

The gene transfer of the tumor suppressor p53 gene has been shown to induce tumor regression in preclinical models. Recent phase I and II studies have been completed in lung cancer with adenoviral-mediated transfer of wild-type p53 (Ad-p53) either alone or in combination with chemotherapy or radiotherapy. These studies have demonstrated acceptable toxicity and evidence of tumor regression with intratumoral delivery of Ad-p53. The predominant clinical effect appears to be locoregional in the area of intratumoral delivery. Further phase III studies are needed to determine if Ad-p53 will play a therapeutic role as a novel agent to treat non-small-cell lung cancer.

PMID: 12044243 [PubMed - indexed for MEDLINE]

Phase II Clinical Trial of Intralesional Administration of the Oncolytic Adenovirus ONYX-015 in Patients with Hepatobiliary Tumors with Correlative *p53* Studies[1]

Della Makower, Alla Rozenblit, Howard Kaufman, Morris Edelman, Maureen E. Lane, James Zwiebel, Hilda Haynes, and Scott Wadler[2]

Division of Hematology/Oncology, Department of Medicine [S. W., M. E. L.], Weill Medical College of Cornell University, New York, New York 10021; Departments of Surgery [H. K.], Radiology [A. R.], Medicine [D. M., H. H.], and Pathology [M. E.], Albert Einstein College of Medicine and the Albert Einstein Cancer Center, Bronx, New York 10463; and Investigational Drug Branch, Cancer Therapy Evaluation Program, National Cancer Institute, NIH, Bethesda, Maryland [J. Z.]

ABSTRACT

Purpose: **ONYX-015 is a genetically modified adenovirus with a deletion of the E1B early gene and is therefore designed to replicate preferentially in *p53*-mutated cells. A Phase II trial of intralesional ONYX-015 was conducted in patients with hepatobiliary tumors to determine the safety and efficacy of such a treatment.**

Experimental Design: **All patients had biopsy-proven, measurable tumors of the liver, gall bladder, or bile ducts that were beyond the scope of surgical resection. Patients received intralesional injections of ONYX-015 at either 6×10^9 or 1×10^{10} plaque-forming units/lesion up to a total dose of 3×10^{10} plaque-forming units, and i.p. injections were allowed in patients with malignant ascites. The status of *p53* was assessed by immunohistochemistry or Affymetrix GeneChip microarray analysis. Studies were conducted for viral shedding and for the presence of antiadenoviral antibodies before and after the injection of ONYX-015. Patients were assessed for response and toxicity.**

Results: **Twenty patients were enrolled, and 19 patients were eligible. Half of the patients had primary bile duct carcinomas. Serious toxicities (> grade 2) were uncommon and included hepatic toxicity (three patients), anemia (one patient), infection (one patient), and cardiac toxicity (one patient, atrial fibrillation). Sixteen patients were evaluable for response. Among these evaluable patients, 1 of 16 (6.3%) had a partial response, 1 of 16 (6.3%) had prolonged disease stabilization (49 weeks), and 8 of 16 (50%) had a >50% reduction in tumor markers. Of the 19 eligible patients, 18 (94.7%) had specimens available for p53 analysis. Fifteen of these 18 patients (83.3%) had evidence of p53 mutation by one or both methods, although the methods correlated poorly. Viral shedding was confined to bile (two of two patients) and ascites (four of four patients). Pretreatment adenoviral antibodies were present in 14 of 14 patients and increased by 33.2% after ONYX-015 treatment.**

Conclusions: **Intralesional treatment with ONYX-015 in patients with hepatobiliary tumors is safe and well tolerated, and some patients had evidence of an anticancer effect. The high incidence of *p53* mutations in these tumors makes this a logical population in which to test this therapy but precludes definitive evaluation about the necessity of a *p53* mutation for ONYX-015 clinical activity.**

INTRODUCTION

ONYX-015 (dl1520, CI-1042) is a type 2, type 5 chimeric Ad[3] that has been genetically modified by disruption of the coding sequence of the M_r 55,000 E1B protein and by insertion of point mutations that generate stop codons in the early coding sequences to prevent the expression of NH_2-terminal protein fragments that might have biological activity (1). These modifications were designed to allow ONYX-015 to replicate preferentially in tumors with defects in the *p53* pathway, which, in addition to mutations in p53 itself, could also include loss of $p14^{ARF}$ function or overexpression of MDM2; the selectivity of this approach derives from the fact that nearly all nonneoplastic tissues have wild-type *p53* (2). ONYX-015 has demonstrated antineoplastic effects *in vitro* against a wide range of human tumor cells, including numerous carcinoma lines with either mutant or normal *p53* gene sequences (3, 4). In a Phase I clinical trial of intralesional injection in patients with head and neck cancer, dose-limiting toxicity was not reached at 1×10^{11} pfu, and ONYX-015 was able to be administered safely, with a predominant toxicity of fevers in 21% of patients (5). In Phase II trials, administered as a single agent (6) or in combination with chemotherapy (7), ONYX-015 has demonstrated clinical activity. There-

Received 6/17/02; revised 9/3/02; accepted 9/10/02.
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[1] Supported in part by Grant N01-CM-07003-74 and Cancer Center Support Grant CA 13330 from the National Cancer Institute, NIH and by a grant from the Chemotherapy Foundation.
[2] To whom requests for reprints should be addressed, at Division of Hematology/Oncology, C606, Weill Medical College of Cornell University, 1300 York Avenue, New York, NY 10021. E-mail: scw2004@med.cornell.edu.

[3] The abbreviations used are: Ad, adenovirus; pfu, plaque-forming unit(s); IHC, immunohistochemistry; CT, computed tomography; ECOG, Eastern Cooperative Oncology Group; FNA, fine-needle aspiration; CR, complete response; PR, partial response; PD, progressive disease; SD, stable disease; CPE, cytopathic effect; PBST, phosphate-buffered saline with 0.1% Tween-20.

fore, it has been proposed that this agent be studied in tumors refractory to conventional therapeutic agents (8).

Cancers of the liver, bile ducts, and gall bladder are an important clinical problem worldwide and will account for more than 17,000 deaths in the United States in 2002 (9). Whereas each has a distinct natural history, etiology, and clinical course, tumors of the liver, bile ducts, and gall bladder share several common features. They are embryologically related (10) and locally aggressive tumors (11), and once beyond the scope of surgical resection, they are generally refractory to nearly all commonly used chemotherapeutic agents (12, 13). Furthermore, these tumors have a high rate of *p53* mutation (14–17), suggesting that they may be attractive candidates for p53-targeted therapy such as ONYX-015.

To investigate this question, we initiated a Phase II trial of ONYX-015 in patients with hepatobiliary tumors to assess the antitumor efficacy and toxicity of this agent. Because these tumors are generally locally invasive, intralesional therapy was administered. The status of *p53* was evaluated by complementary methods, IHC and Affymetrix GeneChip analysis, which uses microarray technology to detect mutations in exons 2–11 of the gene.

Current concerns regarding therapy that uses a replicating Ad include the risk of infection to patient contacts, especially those who are immunosuppressed, and the potential futility of using an Ad in patients who already have antiadenoviral antibodies. To address the first question, we collected body fluids and measured viral shedding at various time points after viral injection. To address the second question, antiadenoviral antibody levels were measured before treatment and again at 1–3 weeks after injection. Our results demonstrate that intralesional injection of ONYX-015 can be administered safely in patients with hepatobiliary tumors with evidence of antineoplastic effects despite the presence of antiadenoviral antibodies and that the risk of horizontal transmission is probably low. The prevalence of *p53* mutations in these tumors was higher than expected using the combination of IHC and Affymetrix analysis.

PATIENTS AND METHODS

Administrative. This was a single-institution, prospective Phase II trial. The protocol was approved by the Investigational Drug Branch of the Cancer Therapy Evaluation Program (National Cancer Institute, NIH), by the Protocol Review Committee of the Albert Einstein Cancer Center, and by the Institutional Review Board and Biosafety Committees of the Montefiore Medical Center.

Eligibility. Patients were required to have an advanced or metastatic carcinoma of the hepatobiliary system (including hepatocellular carcinoma, cholangiocarcinoma, carcinoma of the gallbladder, or carcinoma of the ampulla of Vater) that was beyond the scope of surgical resection. All patients had measurable disease, accessible by either CT-guided percutaneous needle aspiration or endoscopy, ECOG performance status of 0 or 1, and adequate organ function, defined as a leukocyte count $\geq 3.5/mm^3$, platelets $\geq 100{,}000/mm^3$, serum creatinine $\leq$ 2.0 mg/dl, and aspartate aminotransferase and alanine aminotransferase $\leq 4\times$ the upper limits of normal. Patients with an elevated total bilirubin $> 3\times$ the upper limit of normal due to biliary obstruction by tumor were eligible for this trial. Patients were allowed to have received ≤ 2 prior chemotherapeutic regimens but must have had no treatment for at least 4 weeks before study entry. Patients who were carriers of hepatitis B were excluded from protocol entry to eliminate the theoretical possibility that inactivation of p53 by the hepatitis B X antigen might lead to replication of ONYX-015 in normal liver parenchyma of hepatitis B carriers (18). Other exclusion criteria included replacement of >50% of liver by tumor, coagulopathy that could not be easily corrected to a prothrombin time <15 s, prior malignancy within 5 years (other than resected basal cell carcinoma of the skin), family history of malignancy suggestive of Li-Fraumeni Syndrome, brain metastases, presence of active infection or other uncontrolled comorbid condition, pregnancy or lactation, requirement for immunosuppressive or antiviral (including antiretroviral) medication, and prior treatment with adenoviral vectors. All patients signed an informed consent form approved by the Montefiore Medical Center Institutional Review Board.

Treatment. Patients were admitted to the hospital 1 day before ONYX-015 administration. To prevent abscess formation in lesions injected with ONYX-015 that might undergo rapid necrosis, the first eight patients treated received prophylactic antibiotic coverage with oral erythromycin (1 g every 6 h) and kanamycin (1 g every 6 h), beginning on the day before ONYX-015 administration and continuing for 24 h after treatment. In addition, patients received i.v. ticarcillin/clavulanate (3.1 g every 4 h) and gentamycin (80 mg every 8 h). Patients allergic to penicillin received vancomycin (1 g every 12 h), aztreonam (2 g every 8 h), and metronidazole (500 mg every 6 h). Antibiotics began on the day before ONYX-015 administration and continued for 1 week after treatment. The protocol was subsequently amended to eliminate prophylactic antibiotics, and the remaining patients received antibiotics only in the case of presumed infection.

ONYX-015 was administered intratumorally in the radiology suite under computed tomographic guidance (Fig. 1, *A* and *B*). The dose of drug was fixed and was not adjusted for body surface area, but it was adjusted for the projected volume of the lesions. The first two patients were initially treated with a total dose of 6×10^9 pfu. After these patients demonstrated no significant toxicity, the dose of ONYX-015 was escalated to 1×10^{10} pfu/lesion, with a maximum dose of 3×10^{10} pfu. The volume of injection was one-third of the total tumor volume, delivered into one to three areas of the lesion. Specifically, lesions < 2 cm received one injection, lesions of 2–4 cm received two injections, and lesions > 4 cm received three injections. Patients with malignant ascites could also receive i.p. ONYX-015 at a dose of 1×10^{10} pfu.

Immediately before ONYX-015 treatment, a FNA was performed at the injection site for histological confirmation of tumor at the site (Fig. 1*D*), as well as for p53 analysis by both IHC (Fig. 1*E*) and p53 probe array. Samples for p53 Affymetrix analysis were snap frozen in liquid nitrogen and stored at −80°C. A maximum of three lesions (including ascites) were treated at one time. Patients with more than three injectable lesions were eligible to return 2 weeks after the original treatment to receive treatment of up to three additional lesions.

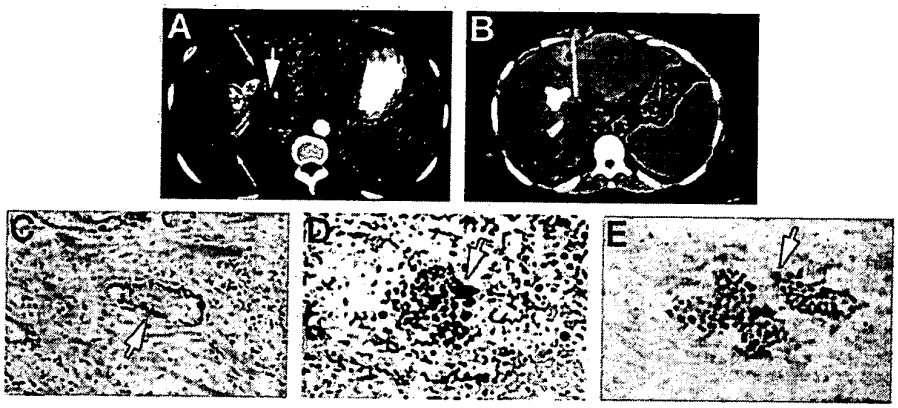

Fig. 1 Administration of ONYX-015 to a patient with a cholangiocarcinoma. *A*, contrast-enhanced CT scan shows a tumor in the porta hepatis (*thick arrow*) extending into the right and left intrahepatic bile ducts. There are multiple biliary stents (*asterisks*) with adjacent bilomas (*thin arrows*). *B*, unenhanced CT scan 1 week later shows a 20-gauge spinal needle used for injection of ONYX-015 into the tumor. *C*, histological specimen demonstrates nests of glandular tumor cells in scirrhous background (*arrow*). *D*, cytologic specimen from the same site as shown in *B* demonstrates the presence of adenocarcinoma (*arrow*). *E*, immunohistochemical staining of specimen from the same site as shown in *B* for p53 demonstrates dark cells that overexpress p53 (*arrow*).

Patients who tolerated treatment and did not develop PD were eligible for retreatment at 3-week intervals.

The first eight patients treated were observed in the hospital on i.v. antibiotics and under contact and droplet isolation protocols for 1 week after receiving ONYX-015. In the absence of both massive hepatic necrosis and evidence of viral shedding, the protocol was amended to allow stable patients to be discharged on the day after ONYX-015 administration.

Samples of urine, sputum, and, when possible, bile and ascites were obtained daily for 1 week after the first ONYX-015 treatment to assess the presence of viral shedding. Serum was also obtained from a subset of these patients before treatment and at 1 and 3 weeks after the first treatment for evaluation of Ad-specific antibodies.

Response Evaluation. Patients were evaluated for response every 6 weeks, by CT scan and tumor markers (CEA, CA19-9, CA125, and, in hepatoma patients, AFP). Radiographic response was defined as follows: CR was defined as disappearance of all radiographic evidence of tumor, normalization of all tumor markers, and stabilization or improvement in performance status, persisting for at least 4 weeks. PR was defined as an at least 50% reduction in the sum of the products of the perpendiculars of all measurable lesions without the appearance of new lesions, stabilization or improvement in all tumor markers, and stabilization or improvement in performance status, persisting for at least 4 weeks. PD was defined as an at least 25% increase in the sum of the products of the perpendiculars of all measurable lesions. Appearance of a new lesion was not considered PD if all injected lesions were stable or responding and the patient was clinically stable. Patients not meeting criteria for CR, PR, or PD were classified as SD.

Because of the difficulty of measuring drug effect on liver lesions and specifically because many of these lesions were highly scirrhous with only minimal nests of tumor cells (Fig. 1*C*; Ref. 11), biochemical assessment of response was also measured. Tumor marker response was defined as a 50% reduction in at least one serum tumor marker that was elevated pretherapy, associated with SD radiographically and stable or improved performance status.

Analysis of *p53* Status. Because ONYX-015 is postulated to replicate only in tumors in which p53 is mutated or in which the p53 pathway is deregulated, *p53* status was assessed by both IHC and Affymetrix GeneChip analysis. Lesions to be injected with ONYX-15 were individually sampled by CT-guided FNA biopsy. A pathologist was present in the radiology suite at the time of FNA to ensure that material procured from radiologically localized lesions was representative of the carcinoma. FNA material from each pass was immediately smeared onto several charged slides, although the number of slides prepared was dependent on the volume of material aspirated. At least one slide prepared from each site was air dried and immediately stained with Diff-Quick stain for microscopic adequacy assessment. If Diff-Quick-stained material was deemed nondiagnostic or insufficient, additional passes from the lesion were requested of the radiologist. All remaining slides were fixed in 95% ethanol and subsequently designated for either *p53* IHC or routine Papanicoloau staining. Slides for IHC were fixed for 20–30 min, air dried, and processed for staining on the same or the subsequent day. IHC staining with p53 antibody (diluted 1:50; DAKO) was performed on the DAKO Autostainer Universal Staining System and developed using the DAKO EnVision$^+$ mouse peroxidase kit with DAKO DAB$^+$ chromogen. Brown nuclear staining of any intensity within tumor cells was interpreted as evidence of p53 immunoreactivity. There was generally insufficient tissue for cellblock preparation, and the limited volume of material obtained also precluded the use of smears as negative controls.

Analysis of p53 by Affymetrix GeneChip. Qiagen DNA Mini kits (Qiagen, Valencia, CA) were used to extract genomic DNA from the same CT-guided FNA used for IHC. These samples were placed on ice at the time of acquisition. The genomic DNA was amplified with PCR using the GeneChip p53

primer set (Affymetrix, Santa Clara, CA) and Amplitaq Gold DNA polymerase (Applied Biosystems, Foster City, CA) according to the Affymetrix instructions for *p53* target preparation. The coding regions of the human *p53* gene were amplified as 10 separate amplicons in a single multiplex reaction. The DNA amplicons were then fragmented using the GeneChip Fragmentation Reagent (Affymetrix) according to the manufacturer's instructions. Fragmented DNA amplicons were labeled at their 3′ ends with a fluoresceinated dideoxynucleotide (fluorescein-ddCTP) and the BioArray terminal labeling kit for DNA probe array assays (Enzo Diagnostics, Farmingdale, NY) according to manufacturer's instructions. Labeled fragments were then placed in hybridization buffer [6× saline-sodium phosphate-EDTA, 0.05% Triton X-100 (Sigma, St. Louis, MO), 2 mg/ml acetylated BSA (Life Technologies, Inc., Grand Island, NY), and 2 nM control oligonucleotide F1 (Affymetrix)]. GeneChip p53 probe arrays (Affymetrix; Ref. 19) were hybridized to labeled fragments and washed on GeneChip Fluidics Station 400 according to the manufacturer's instructions. The probe arrays were scanned (GeneArray Scanner 2508; Affymetrix) and analyzed using the Microarray Suite software version 5.0 (Affymetrix).

Analysis of Viral Shedding. Because of concerns about transmission of ONYX-015 via secretion into body fluids, viral shedding was measured in urine, ascites, bile, and peripheral blood. Initially, attempts were made to collect sputum; however, this was unsuccessful. Two assays were used. In the CPE assay, HEK293 human embryonic kidney cells were grown to 90–100% confluence in a 12.5-cm^3 plug seal flask. Two hundred and fifty μl of patient ascites, bile, urine, or serum were diluted in 200 μl of DMEM. When necessary, pH was adjusted to 7.2–7.4 with 50 μl of sodium bicarbonate [7.5% (w/v)]. Media covering the cells were removed, and 500 μl of the diluted samples were added. The samples were incubated at 37°C with 5% CO_2 for 1 h, with gentle rocking every 20 min. After incubation, 10 volumes (5 ml) of DMEM containing 2.5% fetal bovine serum, 4500 mg/liter glutamine, 100 mIU/ml penicillin, and 100 μg/ml streptomycin were added. Cells were inspected daily for evidence of CPE for up to 14 days. If CPE was apparent, cells were harvested and stored at −80°C. Frozen cells were thawed at 37°C, and cellular debris was removed by centrifugation at 453 × *g* for 15 min. Supernatant was filtered through a 0.22 μm nitrocellulose filter, and 500 μl of the filtered supernatant were then used to reinfect confluent HEK293 cells. After the second infection, 10 volumes (5 ml) of 2.5% fetal bovine serum in DMEM with glutamine, penicillin, streptomycin, and 2.5 μg/ml Fungizone were added to each of the flasks. Where CPE was evident in the second infection, cells were harvested and stored at −80°C until DNA purification. Infection assays were scored positive for ONYX-015 only after verification by PCR. Contents of flasks in which CPE was witnessed after the second infection assay (above) were thawed and centrifuged to remove cellular debris. Supernatant (200 μl) was used with the Qiagen DNA Blood Mini Kit, according to the manufacturer's recommended protocol. DNA was eluted in 35 μl of nuclease-free water and stored at −80°C until PCR was performed. Every effort was made to prevent cross-contamination between samples at all times. For extraction from serum, ascites, and biliary drainage, 200 μl of sample were used directly with the Qiagen kit according to the manufacturer's recommended protocol, with elution in 35 μl of nuclease-free water.

The second assay used nested PCR to detect virus. PCR of purified viral DNA was performed with primers specific for the ONYX-015 virus. Both primer pairs are specific for a region of salmon sperm DNA inserted into the virus during its generation. This region is present on the plasmid dl309, which was used as a positive control for the PCR. The following primers were used to amplify a 585-bp region of the salmon sperm DNA: Int309S, ctgctgccatgttgtgttgctaccat; and Int309AS, acctaccgggaagtcatcaaatgaac. PCR was carried out in 50 μl containing 1× Taq buffer (Eppendorf), 1× Taq enhancer, 100 nM deoxynucleotide triphosphates, 100 nM each primer, 1.7 units of Taq polymerase (Eppendorf), and 10 μl of purified viral DNA as template. The reactions were carried out at 95°C for 3 min, followed by 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min for 35 cycles, and concluded with a 10-min extension at 72°C. Two μl of the initial PCR product were used as a template in a nested PCR reaction. Conditions for the nested PCR were the same as those for the first-round PCR. The following primer pair was used to amplify an internal 335-bp fragment of DNA: ne309S, cctttgcgttctggtaggccg; and ne309AS, ctgtgaagagcacagggcgcc. Gel electrophoresis was performed on the second-round PCR product. Thirty μl of sample were run on a 0.8% agarose gel for detection of transcripts.

Detection of Ad-specific Antibodies by ELISA. ONYX-015 was diluted in PBS and stored overnight at 4°C at 1×10^8 particles/well. Wells were washed five times with PBS + 0.1% Tween 20 and blocked for 1 h in PBST + 1% BSA at 37°C. Serial dilutions of serum samples were made in PBST + 0.1% BSA and added to wells in triplicate. After a 2-h incubation at 37°C, wells were washed three times, and antihuman horse radish peroxidase antibody (Promega) was added at a dilution of 1:10,000. After a 1-h incubation at 37°C, wells were washed three times, and O-phenylenediamine substrate was added. Fifteen min later, absorbance at 450 nm was read on a Bio-Rad Model 550 microplate reader.

Statistical Analysis. Sample size was calculated based on Simon's "minimax" design (20). Standard ECOG response criteria were used to measure response to treatment (21). In the first stage, 13 patients were accrued. If no patients respond, the study is closed, and a response rate of 20% is ruled out with 95% confidence. If at least one response is observed among the first 13 patients, 14 additional patients are accrued. If the true response rate is at least 20%, this would be detected with 95% confidence at a power of 80%.

RESULTS

Patient Demographics. Based on the planned statistical design of the trial, there was one responder among the first 14 patients, and the plan was to continue the trial to 36 patients; however, the trial was terminated administratively because of drug shortage. As shown in Table 1, 20 patients were enrolled. Half of the patients had bile duct tumors (cholangiocarcinoma or carcinoma of the ampulla of Vater). Most of the patients were previously untreated, and all were ambulatory. For one patient, who never received treatment, the diagnosis could not be his-

Table 1 Demographics

	n
Enrolled patients	20
Eligible patients	19[a]
Evaluable patients	16
Male:female	11:9
Age (yrs)	
Median	60
Range	34–78
ECOG performance status 0:1	5:15
Site of primary lesion	
Cholangiocarcinoma	9
Gall bladder carcinoma	5
Hepatocellular carcinoma	5
Carcinoma of the ampulla of Vater	1
Prior chemotherapy 0:1:2	12:6:2

[a] No tissue confirmation in one patient.

Table 2 Toxicities (n = 19)

	National Cancer Institute common toxicity criteria grade				
	0	1	2	3	4
Leukopenia	17	1	1	0	0
Anemia	17	0	1	1	0
Thrombocytopenia	16	3	0	0	0
Fever	6	4	9	0	0
Myalgias	10	6	3	0	0
Abdominal pain	12	7	0	0	0
Infection	18	0	0	0	1
Nausea/vomiting	16	1	2	0	0
Hepatic	14	1	1	2	1
Cardiac	18	0	0	1	0
Arthritis	18	0	1	0	0
Hypertension	18	0	1	0	0
Hypotension	18	0	1	0	0

tologically confirmed at the planned site of injection, and this patient was therefore ineligible. Among the remaining 19 patients, 49 cycles (mean, 3 cycles/patient; range, 0–6 cycles/patient) of therapy were administered. The median number of lesions treated was 3 (range, 0–6 lesions). In addition, four patients received i.p. therapy for malignant ascites.

To confirm the safety and tolerability of ONYX-015 intralesional therapy, a planned dose escalation was incorporated into the study design. The first two patients received 6×10^9 pfu/lesion, and the same patients were subsequently escalated to a dose of 1×10^{10} pfu/lesion with a maximum dose of 3×10^{10} pfu. The remaining patients received 1×10^{10} pfu/lesion with a planned maximum dose of 3×10^{10} pfu, which was delivered to all subsequent patients.

Toxicities. As shown in Table 2, among the 19 eligible patients, therapy was well tolerated. The one episode of cardiac toxicity was atrial fibrillation, which was asymptomatic and required medical therapy only. In retrospect, this patient's atrial fibrillation had likely predated her treatment with ONYX-015. Hepatic toxicity generally resolved spontaneously when related to therapy or, alternatively, was secondary to disease progression. No patients had sequelae from altered liver functions, and the single incidence of grade 4 hepatic toxicity was related to disease progression rather than viral-induced liver failure. Likewise, the single grade 4 infection was related to tumor progression resulting in sepsis. Postinjection fever, chills, and myalgias were frequent but mild and self-limited. Hematological toxicity was also mild. There were no complications resulting from the injection, including bleeding or infection.

Table 3 Response to treatment (n = 19)

	n
CR	0
PR	1
Reduction in tumor markers by >50%	8
SD	12
PD	3
Not evaluable	3

Response to Treatment. Among the 19 eligible patients, 16 were evaluable for response to therapy. Three patients died before completing their first response assessment of causes felt to be unrelated to study treatment. The first, a 74-year-old man with metastatic cholangiocarcinoma and portal hypertension, died of a variceal bleed 10 days after receiving ONYX-015 treatment. The second, a 43-year-old woman with cholangiocarcinoma, died of a cerebrovascular accident 22 days after receiving ONYX-015. The third patient, a 43-year-old woman with cholangiocarcinoma, developed sudden onset of hypothermia, hypotension, and metabolic acidosis 14 days after ONYX-015 treatment and died of presumed sepsis 24 h later. No causative organism was identified.

Among the 16 patients evaluable for response, there was one (6%) PR in an injected lesion lasting 13.5 weeks (Table 3). In addition, 8 of 16 (50%) evaluable patients had declines of at least 50% in at least one serum tumor marker, associated with SD radiographically, and stable or improved performance status. These "tumor marker responses" lasted for a median of 11.5 weeks (range, 6.5–20.5 weeks). One additional patient, who had recurrent cholangiocarcinoma, exhibited a prolonged period of SD (radiographically, clinically, and via tumor markers) for 49 weeks.

Analysis of *p53* Status. Among the 19 evaluable patients, *p53* status was analyzed by two methods. A positive signal on IHC indicates the presence of a mutation in the genome, which stabilizes the protein and increases the normally short half-life. This methodology was used and was informative in 17 of the 19 patients, of whom 9 (52.9%) exhibited a positive signal for *p53* (Table 4). Affymetrix GeneChip uses microarray technology to analyze the entire coding region of *p53* (exons 2–11). The microarray assay identifies missense mutations and single-base deletions and can identify mutant *p53* in a background of wild-type *p53*. Eighteen of 19 patients had DNA samples that were informative by GeneChip analysis. Of these, 11 of 18 (61.1%) demonstrated mutations in *p53* (Table 4). When patient samples were analyzed for mutation in *p53* by either IHC or Affymetrix, 15 of 18 (83.3%) samples were positive. Nevertheless, there was poor correlation between the techniques: only 7 of 17 (41.1%) samples analyzable and informative for both techniques were concordant (5 samples were positive by both techniques, and 2 samples were negative by both techniques).

Table 4 Correlation of response with *p53* status

Patient no.	Eligible	Evaluable	Primary	OR[a]	TMR	IHC	Affy	Either	Concordance
1	Y	Y	Chol	N	Y	Y	Y	Y	Y
2	Y	Y	Chol	N	NE	N	N	N	Y
3	Y	Y	Chol	N	N	Y	N	Y	N
4	Y	N	Chol	NE	NE	N	Y	Y	N
5	Y	Y	Chol	N	N	Y	N	Y	N
6	Y	Y	Chol	N	N	N	Y	Y	N
7	Y	Y	Ampulla	N	Y	N	Y	Y	N
8	Y	Y	GB	N	Y	Y	Y	Y	Y
9	Y	Y	GB	N	Y	N	Y	Y	N
10	Y	Y	Hep	N	N	N	Y	Y	Y
11	Y	Y	Hep	Y	N	Y	N	Y	N
12	N	N	Hep	NE	NE	NE	NE	NE	NE
13	Y	Y	Hep	N	Y	NE	N	N	NE
14	Y	N	Chol	NE	NE	Y	Y	Y	Y
15	Y	N	Chol	NE	NE	Y	N	Y	N
16	Y	Y	Chol	N	Y	NE	NE	NE	NE
17	Y	Y	GB	N	Y	Y	Y	Y	Y
18	Y	Y	GB	N	N	N	Y	Y	N
19	Y	Y	GB	N	N	Y	Y	Y	Y
20	Y	Y	Hep	N	Y	N	N	N	N
Totals	19/20	16/19		1/16	8/15	9/17	11/18	15/18	7/17

OR, objective response; TMR, tumor marker response; Affy, Affymetrix GeneChip analysis; Either, either IHC or Affymetrix analysis positive for p53 mutation; Concordance, both assays provide same results; Chol, cholangiocarcinoma; Ampulla, carcinoma of the ampulla of Vater; GB, gall bladder carcinoma; Hep, hepatocellular carcinoma; NE, not evaluable; Y, yes; N, no.

Correlation of *p53* Status with Response. As shown in Table 4, it is difficult to correlate *p53* status with response because 83.3% of patients had evidence of *p53* abnormalities by at least one technique. However, it is interesting to note that the only radiographic response to this agent was seen in patient 11, who had only a borderline positive signal for p53 overexpression (<1%) by IHC and no evidence of mutation by Affymetrix analysis. Patient 2, who had prolonged stabilization of disease, had no evidence for mutation in *p53*. Among the eight tumor marker responders, seven (patients 1, 7, 8, 9, 13, 17, and 20) had specimens informative for p53 status by at least one method (six of eight specimens were informative for *p53* status by IHC, and seven of eight were informative for p53 status by Affymetrix). Five of seven (71.4%) responders had a p53 mutation by at least one method (three of six had a *p53* mutation by IHC, and six of seven had a *p53* mutation by Affymetrix). Among the six evaluable patients who were neither tumor marker responders nor had a PR or prolonged stabilization (patients 3, 5, 6, 10, 18, and 19), six of six had a *p53* mutation by one or both methods (three of six had a *p53* mutation by IHC, and four of six had a p53 mutation by Affymetrix).

To account for the absence of concordance between IHC and Affymetrix analysis, specific mutations were analyzed (Table 5). In patient 1, *p53* was overexpressed, and a Q167E missense mutation was observed in a codon within the zinc-binding domain by microarray analysis. In patient 2, no aberrancies in *p53* were observed by either technique. In patients 3, 5, and 15, no mutations were observed by microarray analysis, but overexpression of *p53* was observed by IHC. In patients 4 and 6, null mutations in regions before the p53 tetramerization domain, which were therefore likely inactivating mutations, were detected by microarray analysis in the absence of overexpression by IHC.

Patient 7 had three missense mutations, two within the S2-S2′ β strand, with no overexpression of *p53* by IHC. In patients 8 and 18, both a polymorphism and a missense mutation outside of a conserved region of the *p53* genome were observed; in patient 8, this was associated with overexpression of *p53* by IHC, but in patient 18, overexpression of p53 by IHC was not observed. Patient 9 had four missense mutations by microarray analysis in the absence of overexpression of *p53* by IHC. Two mutations, S241C and R273P, were in bases that directly contact DNA, and one mutation, E286Q, was in the H2-helix region of the molecule. Patient 10, who was also without overexpression of *p53*, had a polymorphism and an E286Q mutation also in the H2-helix region. Patient 11 had no mutations detectable by microarray analysis and had borderline p53 overexpression ($0.7 \pm 1.1\%$) by IHC.

Patient 14 had two missense mutations, one in conserved region IV and an R273P mutation in the *p53* DNA binding region. Patient 17 had two missense mutations, one of which was also in region IV. Patient 19 had a sole missense mutation in R273P. Both patients also had overexpression of *p53* by IHC.

Patients with either a missense or null mutation in p53 by microarray analysis or overexpression of *p53* by IHC were considered to have a mutation. Only two patients (patients 2 and 20) demonstrated wild-type *p53* by both techniques. Patient 11, who had a negative microarray analysis and overexpression of *p53* in <1% of cells analyzed, was considered borderline positive.

Antiadenoviral Antibody Studies. The presence of Ad-specific antibody levels was evaluated by ELISA before ONYX-015 treatment in the first 14 patients enrolled on trial. All patients demonstrated Ad-specific antibodies. Eight of these patients were also evaluated for Ad-specific antibodies at 1–3 weeks after their first treatment. Antibody levels increased by a

Table 5 Correlation of *p53* mutation by IHC and Affymetrix analysis

Patient no.	Microarray exon	Mutation	Nucleotide position	AA change	IHC
1	Exon 5	ctg>ctg	517	L145R	13.7% (±2.1)
	Exon 5	atg>ctg	573	M160L	
	Exon 5	cag>gag	604	Q167E	
	Intron 10	g>a	1368		
2	0	0			0
3	0	0			19.6% (±9.4)
4	Exon 6	cga>tga	724	R196	0
5	0	0			12.4% (±5.3)
6	Exon 8	cga>tga	1146	R306	0
	Intron 5	a>g	687		
7	Exon 5	caa>cac	488	Q136H	0
	Exon 5	gcc>ccc	492	A138P	
	Exon 5	ccc>ccg	542	P151	
8	Exon 6	cga>cgg	801	R213	11.4% (±5.4)
	Exon 6	gtg>ctg	814	V217L	
9	Exon 7	tcc>tgc	906	S241C	0
	Exon 8	cgt>cct	1027	R273P	
	Exon 8	ggg>gcg	1053	G279A	
	Exon 8	gaa>caa	1080	E286Q	
10	Exon 4	ccg>ccc	166	P36	0
	Exon 8	gaa>caa	1080	E286Q	
11	0	0			0.7% (±1.1)
12	NE[a]	NE	NE	NE	NE
13	0	0			NE
14	Exon 7	atg>atc	925	M246I	61%
	Exon 8	cgt>cct	1027	R273P	
	Intron 10	a>g	1367		
15	0	0			14.2% (±8.9)
16	NE	NE	NE	NE	NE
17	Exon 7	atc>aac	956	I254N	95%
	Exon 8	ggg>gcg	1135	G302A	
18	Exon 6	cga>cgg	801	R213	0
	Exon 6	cat>gat	803	H214D	
19	Exon 8	cgt>cct	1027	R273P	Skin nodule: 84%, Periumbilical nodule: 58%
	Intron 8	g>t	1160		
20	Intron 6	g>a	838		NE

[a] NE, not evaluable.

mean of 33.2 ± 10.3% after treatment, consistent with other trials (22). No relationship between tumor marker decline and Ad-specific antibodies (either pretreatment level or posttreatment increase) was observed.

Cell-mediated immune response was not formally measured; however, in one patient with a biliary tumor who died, the autopsy specimen revealed dense lymphoid aggregates in the area of lymphatic drainage for the injection site (Fig. 2). No Ad was detectable by immunohistochemical staining in either the primary tumor or the lymphatic drainage in the liver.

Analysis of Viral Shedding in Body Fluids. Posttreatment samples of serum, urine, and, where possible, ascites and biliary fluid were obtained from the first 14 patients enrolled on trial and assayed for viral shedding by CPE assay and PCR. Originally, we had planned to assay patient sputum; however, this strategy had to be abandoned due to the negligible amount of sputum produced by patients in the postinjection period.

Fifty-five samples of patient urine were obtained from 12 patients at various time points between 1 and 14 days postinjection of ONYX-015 and analyzed for viral shedding by CPE assay. No specimen showed any evidence of viral shedding. PCR was not performed on urine samples due to technical difficulties with the procedure. Two patients had biliary stents available for analysis of shedding into bile. Both had evidence of viral shedding into bile by PCR analysis at 1–5 days after injection of ONYX-015. Of the four patients with ascites, two had positive CPE assays at 1–6 days, and all four had evidence of virus by PCR analysis at 1–9 days postinjection.

DISCUSSION

The most important finding of this study was that ONYX-015 can safely be administered intralesionally in patients with hepatobiliary tumors and that some patients had evidence of therapeutic effect as measured by radiological response, prolonged stabilization of disease, or a ≥50% decrease in levels of tumor markers. Given the highly scirrhous histology of these tumors (Fig. 1), biochemical evidence of response by measurement of declines in tumor markers may be a reasonable alternative to radiographic measurement of response, although by no means are the two equivalent. The lack of serious toxicities in this population and the findings of a favorable clinical outcome in about half of the patients suggest that intralesional therapy

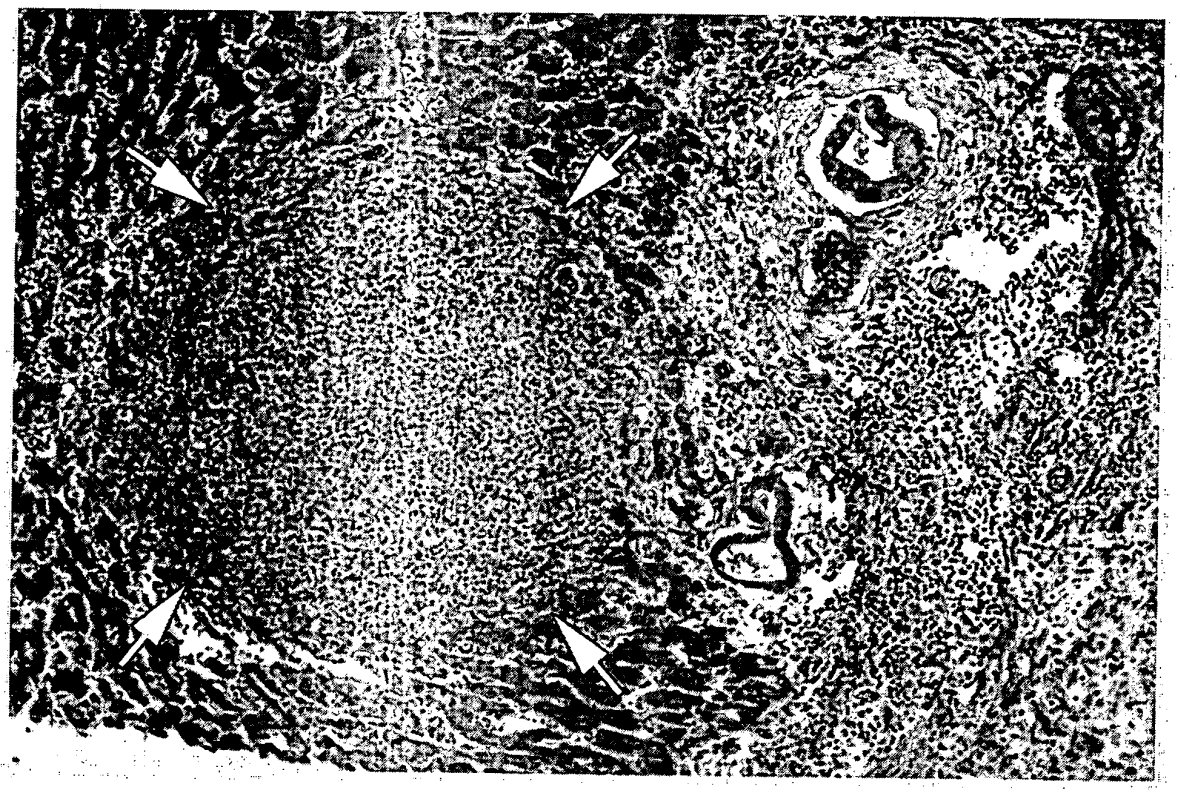

Fig. 2 Tissue section from autopsy specimen of a patient with a biliary tumor. The section shows normal liver with robust lymphocytic aggregation (*arrows*) at the site of the lymphatic drainage from the biliary tumor to the liver. No virus was present on routine immunohistochemical staining.

with ONYX-015, despite the cumbersome nature of the CT-guided injections, is nevertheless worth exploring further either as a single-agent therapy or in combination with cytotoxic agents for which there is evidence for synergy (3, 7, 22–25).

Other than surgery or liver transplantation, there are few effective therapeutic options for patients with hepatobiliary tumors. One small trial of ONYX-015 (dl1520) injection in patients with hepatocellular carcinoma suggested some modest level of clinical benefit with minimal toxicities (27). Because most of the studies with ONYX-015 have been performed in patients with head and neck, ovarian, or pancreatic cancer, our trial was novel and suggests a clinical benefit in hepatobiliary tumors. Additional studies should be considered in this group of patients.

Despite the presumed selectivity of the virus for *p53*-mutated cells (essentially excluding normal tissues), the widespread prevalence of antiadenoviral antibodies in the normal population, and the benign nature of adenoviral infection, horizontal transmission of virus is a concern with ONYX-015 therapy. The viral shedding studies, although limited, are therefore encouraging. Whereas we were unable to obtain adequate sputum for viral studies, the absence of viral shedding in the urine suggests effective systemic clearance of the virus. One concern, however, is the presence of virus in the bile; additional studies with stool samples may be warranted. Shedding into ascites was also of interest, not in terms of horizontal transmission, but in terms of potential for antineoplastic effects against i.p. tumors.

The nearly universal presence of antiadenoviral antibodies in our population demonstrates that the therapeutic effect of ONYX-015 is not necessarily compromised by intact humoral immunity, although in the absence of a comparable group of patients without preexisting antiadenoviral antibodies, it is not possible to definitively exclude an antibody-mediated decrement in the clinical activity of the virus. The efficacy of our approach may relate to the intralesional route of injection, which may compartmentalize the virus at least temporarily from the immune response. Alternatively, the ability of ONYX-015 to enter and replicate in tumor cells without immediately lysing them may create a sanctuary for viral replication within the p53-mutated or possibly even the p53 wild-type tumor cells. Thus, the bolus injection of concentrated virus directly into tumors may allow binding and entry of virus into tumor cells before the immune response can be mobilized, and virus may subsequently replicate intracellularly, spared from humoral or cell-mediated immune response. Data generated using *in situ* RT PCR analysis of ONYX-015 injected into two abdominal wall implants support this hypothesis (26).

One controversial question regarding the utility of ONYX-015 is the degree of selectivity for *p53*-mutated cells. There is clear evidence that ONYX-015 can replicate in cells with wild-type *p53* (3, 25, 28, 29). Others have argued, however, that the *p53* pathways in these cells may be disrupted because of decreased levels of $p14^{ARF}$ or elevated levels of MDM2, which

could functionally inactivate *p53* (30–32). There is also evidence that ONYX-015 can infect cells regardless of p53 status but can replicate more efficiently in *p53*-mutated cells (33).

To investigate the status of *p53* in the patients enrolled in this trial, two methods of mutation detection were used, microarray analysis using the Affymetrix Genechip, which has provided good agreement with direct manual sequencing (34), and IHC. A principal finding of our study was the absence of correlation between the microarray analysis and the IHC analysis. Six patients with mutations detected by microarray analysis had no overexpression of *p53* by IHC. Three had null mutations located before the tetramerization domain and would not be expected to produce functional proteins. Because IHC detects mutant proteins based on epitope recognition, truncated proteins may be missed with this method. The other three cases are more difficult to explain. Two patients had missense mutations in codons for amino acids in direct contact with the DNA binding domain or in the H2-helix regions, which are likely inactivating lesions. This is confirmed by clinical studies in which patients with missense mutations in the protein-DNA binding domain, H2-helix, or loop structures had a substantially worse prognosis than patients with wild-type *p53* (35). The fifth patient had both a polymorphism and a missense mutation outside a conserved region and had no overexpression of *p53* by IHC. These patients also tend to have a worse prognosis than patients with wild-type p53 but have an improved prognosis when compared with patients with mutations in the conserved regions (35). There were also three patients with overexpression of *p53* ranging from 12.4–19.6% of analyzed cells, who did not have mutations detected by microarray analysis. Because the GeneChip algorithm is not optimized for detection of intragenic deletions or insertions, this is the likely explanation for this observation (36).

One interesting finding from this study is that 3 of 18 patients had identical g→c mutations in codon 273, substituting a proline for an arginine. This R273P mutation was of interest for several reasons: (*a*) mutations at codon 273 are the most common mutations in *p53*; (*b*) the arginine at 273 is one of the amino acids in direct contact with DNA in the DNA-protein binding region of *p53*; and (*c*) the fact that this mutation occurred in nearly 19% of patients, a rate that appeared to be higher than that which would have occurred by chance. Two databases of *p53* mutations, one from the WHO, which listed 15,329 mutations (IARC TP53 mutation database;[5] IARC, WHO, Lyon, France), and one from the Institut Curie, which listed 14,969 mutations (T. Soussi, C. Gallou, and C. Beroud, p53 database,[6] Laboratoire de Genatoxicologie des Tumeurs, Institut Curie, Paris, France) failed to correlate the R273P mutation with tumors of the liver, gall bladder, or bile duct, and several studies of patients with hepatobiliary tumors failed to demonstrate the presence of this mutation (37–39). This observation may indicate a novel *p53* mutation but will require confirmation by direct sequencing.

In summary, our study showed that intralesional injection of ONYX-015 in patients with hepatobiliary tumors was safe and well tolerated. Over half of the patients had some evidence of clinical benefit, despite the universal presence of antiadenoviral antibodies before treatment. Although limited, shedding studies showed no risk for horizontal transmission of virus, making this therapy applicable to the clinic. Finally, mutations in *p53* were detected in >80% of patients who received treatment by at least one of two methods, making any decision about the necessity of p53 mutation for viral replication moot. The absence of concordance between IHC and the Affymetrix GeneChip array analysis was a concern for future studies, and neither may provide the ultimate answer (40). Additional studies to explore the best way to analyze *p53* are warranted.

[5] www.iarc.fr/p53/index.html.

[6] www.p53.curie.fr.

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Repeated intravesical instillations of an adenoviral vector in patients with locally advanced bladder cancer: a phase I study of p53 gene therapy.

Pagliaro LC, Keyhani A, Williams D, Woods D, Liu B, Perrotte P, Slaton JW, Merritt JA, Grossman HB, Dinney CP.

Department of Genitourinary Medical Oncology, Box 427, The University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030-4009, USA. lpagliar@mdanderson.org

Related Resources

PURPOSE: We investigated the feasibility, safety, and biologic activity of adenovirus-mediated p53 gene transfer in patients with locally advanced bladder cancer. PATIENTS AND METHODS: Patients with measurable, locally advanced transitional-cell carcinoma of the bladder who were not candidates for cystectomy were eligible. On a 28-day cycle, intravesical instillations of INGN 201 (Ad5CMV-p53) were administered on days 1 and 4 at three dose levels (10(10) particles to 10(12) particles) or on either 4 or 8 consecutive days at a single dose level (10(12) particles). RESULTS: Thirteen patients received a total of 22 courses without dose-limiting toxicity. Specific transgene expression was detected by reverse transcriptase polymerase chain reaction in bladder biopsy tissue from two of seven assessable patients. There were no changes in p53, p21waf1/cip1, or bax protein levels in bladder epithelium evident from immunohistochemical analysis of 11 assessable patients. Outpatient administration of multiple courses was feasible and well tolerated. A patient with advanced superficial bladder cancer showed evidence of tumor response. CONCLUSION: Intravesical instillation of Ad5CMV-p53 is safe, feasible, and biologically active when administered in multiple doses to patients with bladder cancer. Observations from this study indicate that this treatment has an antitumor effect in superficial transitional-cell carcinoma. Improvements in the efficiency of gene transfer and the levels of gene expression are required to develop more effective gene therapy for bladder cancer.

Publication Types:
- Clinical Trial
- Clinical Trial, Phase I

PMID: 12805322 [PubMed - indexed for MEDLINE]

1: Curr Opin Drug Discov Devel. 2003 Mar;6(2):169-73. Related Articles, Links

Development of vaccines against self-antigens: the p53 paradigm.

Chada S, Mhashilkar A, Roth JA, Gabrilovich D.

Introgen Therapeutics Inc, 2250 Holcombe Boulevard, Houston, TX 77030, USA. s.chada@introgen.com

Active immunotherapy using dendritic cells (DCs) to deliver tumor antigens has generated considerable excitement among oncologists worldwide. Although most tumor antigens used in immunotherapeutic approaches are tumor-associated, often, little is known about the underlying biology of the target. Here, we review the use of 'obligate' tumor antigens, where antigen expression is a prerequisite for tumor formation or maintenance. The prototype for this class of antigens is the p53 tumor antigen, which is mutated in > 50% of human malignancies. The direct involvement of p53 in the malignant transformation of tumors makes it an attractive target for immunotherapy. p53-Reactive antibodies have been found in patients with various types of cancer, demonstrating that the human immune system can recognize and respond to tumor-associated p53. Extensive preclinical experimentation has now validated the translation of p53-expressing DCs into a clinical setting. Clinical trials are ongoing to evaluate the safety and antitumor responses elicited by DCs transduced with adenoviral-p53 in cancer patients.

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- Review
- Review, Tutorial

PMID: 12669451 [PubMed - indexed for MEDLINE]